Prospects for using Entomopathogenic Nematodes as a Biocontrol Agent against Fungus gnats, *Bradysia* spp. (Diptera: Sciaridae) in nursery and glass house crops

by

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Declaration

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Abstract

Fungus gnats of the genus *Bradysia*, have become important pests to undercover, cultivated crops. Commonly referred to as mushroom fly, nuisance flies, or dark-winged flies, they have a global distribution. Their larval stage, which is the main feeding stage of the insect, feeds on the radical system of the plant, as well as tunnels into the stems, resulting in direct physical damage to the plant. Coupled herewith is the indirect damage that is caused through the creation of entry points for soil-borne pathogens. In South Africa, *Bradysia impatiens*, which is one of the most important species of fungus gnats that specialises in feeding on living plants, has recently been identified in association with major tree nursery beds. In the course of this study, the same species, *B. impatiens* was found to be living in association with greenhouse-grown cucumbers, mushrooms, blueberries, tomatoes, herbs, and chrysanthemums. *Bradysia impatiens* was concluded as being a well-established pest species in South African undercover farming systems. *Lycoriella sativae* was reported for the first time, as a pest on mushroom, for South Africa, and the Afro tropical region.

The use of entomopathogenic nematodes (EPNs) for the control of *Bradysia* spp. has proved to be relatively advantageous, since sciarids are mainly pests of protected crops that are either too close to harvest, or for which, otherwise, the application of pesticides is unsuitable. The control of *Bradysia* spp. using EPNs has been dominated by the use of *Steinernema feltiae*, which, despite having a global distribution, has, to date, not yet been isolated either from South Africa, or from the rest of the African continent, except for Algeria. *Steinernema feltiae* has also been cited as being biologically constrained at relatively high temperatures, corresponding to most greenhouse temperatures in South Africa. The objective of this study was to identify the *Bradysia* spp. causing problems in greenhouses in the Western Cape province, as well as to test locally isolated EPN species for their potential to control *Bradysia* spp.

Using molecular techniques and morphological observations, the species involved were identified as *Bradysia impatiens* Johannsen and *Lycoriella sativae* Johannsen. A laboratory culture for *B. impatiens* was established using a 3:1:1 mixture of pine sawdust, cornmeal and soy meal. The number of eggs laid per single adult female was between 100 and 250. A new phenomenon of egg laying, in the form of chains, was observed for the first time, for *B. impatiens*. In length, the first instar larvae measured between 1.2 and 2.5 mm, the second instars between 2.6 and 4.5 mm, and the third instars between 4.6 and 6.5 mm, while the fourth instar larvae measured about 6.5 to 7.2 mm. The life cycle of *B. impatiens* lasted for approximately three weeks in a growth chamber, at 25°C in the dark.

Eight South African local EPN species, and a foreign *S. feltiae*, were tested for their ability to kill fourth instar larvae of *B. impatiens*. Bioassay protocols were performed to determine the insecticidal activity, the lethal dose, and the efficacy of the EPNs at different temperatures. The results showed four local EPN species, *Steinernema yirgalemense, Heterorhabditis noenieputensis*, *Heterorhabditis indica*, and *Heterorhabditis zealandica*, achieving higher than 80% mortality, at 25°C and 30°C, from the different bioassays performed. In contrast, *S. feltiae* achieved higher percentage mortality at 13°C, (82%), at which temperature, none of the local isolates was able to infect fourth instar larvae of *B. impatiens*. *Steinernema feltiae* maintained a high percentage mortality at 25°C, (72%) but registered a much lower mortality for *B. impatiens* at 30°C, (47%). *Heterorhabditis zealandica* had the lowest LD_{50} of 2.6 infective juveniles (IJs) per larva, and an LD_{90} of 18.68 IJs/larva, while *S. yirgalemense* and *H. indica* had LD_{50} and LD_{90} of 9; 64, and 9; 64, respectively. *Steinernema yirgalemense, H. indica* and *H. zealandica* were all able to reproduce inside the fourth instar larvae of *B. impatiens*, and to produce a new cohort of IJs. The relatively bigger nematodes, *S. jeffreyense*, *S. khoisanae*, and *Steinernema* sp. (WS9), were unable to infect the fourth instar larvae of *B. impatiens*, which showed that the size of the EPNs affected their ability to infect fungus gnat larvae. The locally isolated EPNs were concluded as having high potential for use against fungus gnats in South Africa.

The effect of different concentrations of a local entomopathogenic nematode, *S. yirgalemense,* for its potential to control a natural infestation of *B. impatiens* in a commercial cucumber greenhouse was tested. Additionally, the effect of potting media on nematode movement to control an artificial population of fungus gnats was tested. The concentrations used were based on the recommended concentration of *S. feltiae* (a commercial product) for the control of sciarids (5×10^5 IJs per m^2). Doubling the number of nematodes in the recommended dosage reduced the number of fungus gnats by 77% after 14 days, and by 76% after 21 days. However, the fungus gnat populations were observed to restore quickly to their original levels, after a period of approximately three weeks, emphasising the short life cycle of the fly, as well as the importance of the pre-treatment, and of consecutive applications, of EPNs throughout the whole production process. The three different types of potting media, consisting of pine sawdust, coco coir, and a mixture of both bale coir and vermiculite, all had a positive effect on the infection of *S. yirgalemense.* Mortality of >75 % was achieved for the fungus gnat larvae that were added to the media, as indicated by the number of adult fungus gnats caught on the yellow sticky cards used.

The results of the current study show that, under optimum conditions, the locally isolated EPNs, namely *H. zealandica, S. yirgalemense*, and *H. indica*, have great potential for use as biocontrol agents for *B. impatiens*. However, additional research should be undertaken into the effect of timing, concentrations, and nematode application techniques, among other factors, so as to obtain further critical information regarding the field applications of the nematodes. Further studies should look at different factors that might affect the optimum field performance of *S. yirgalemense*. The factors involved could include the pre-treatment of the substrates, and the use of higher concentrations of EPNs, and follow-up applications, the timely applications of EPNs, and other factors, such as pre-treatment with cadaver application, versus the aqueous drench application of the EPN species. However, the application of nematodes should not be seen as a stand-alone, but it

should, rather, be implemented as part of an integrated pest management system, with sanitation being the most important consideration.

Opsomming

Muggies van die genus *Bradysia,* het ’n ernstige plaag geword wat bedekte, verboude gewasse betref. Hulle staan ook as donkervlerkswammuggies bekend en kom wêreldwyd voor. Gedurende die larvale fase, wat die insek se hoofvoedingsfase is, voed dit op die plant se wortelstelsel en boor dit in die stamme in, wat tot fisiese skade aan die plant lei. Gepaardgaande indirekte skade word deur die skepping van toegangspunte vir grondgedraagde patogene veroorsaak. In Suid-Afrika is *Bradysia impatiens*, een van die belangrikste muggiespesies wat daarin spesialiseer om lewende plante te voed en is onlangs in assosiasie in boomkwekerye geïdentifiseer. In die verloop van hierdie ondersoek is daar bevind dat *B. impatiens* in assosiasie met kweekhuiskomkommers, sampioene, bloubessies, tamaties, kruie en krisante voorkom. *Lycoriella sativae* is vir die eerste keer aangemeld as ’n plaag van sampioene vir Suid-Afrika en die Afro tropiese streek.

Daar is reeds getoon dat die gebruik van entomopatogenies nematodes (EPNs) betreklik voordelig is in die bestryding van *Bradysia*, aangesien die Sciaridae meestal ’n plaag by beskermde gewasverbouing is, wat óf byna geoes word óf waarvoor die gebruik van plaagdoders andersins nie moontlik is. Die beheer van *Bradysia* met behulp van EPNs word deur die gebruik van *Steinernema feltiae* oorheers, wat, ten spyte van wêreldwye verspreiding, tot op datum nog nie in Suid-Afrika of in die res van die vasteland geïsoleer is nie, behalwe in Algerië. Daar word genoem dat *S. feltiae* biologies minder effektief is met betreklik hoë temperature, wat ooreenstem met die meeste kweekhuise temperature in Suid-Afrika. Die doel van hierdie studie was om die *Bradysia* spesies te identifiseer wat probleme in kweekhuise in die Wes-Kaap skep en om plaaslik geïsoleerde EPN-spesies te toets vir hul potensiaal om *Bradysia* te bestry.

Deur molekulêre tegnieke en morfologiese waarnemings te gebruik, is die spesie as *B. impatiens* geïdentifiseer. ’n Laboratoriumteling is met behulp van ’n 3:1:1-mengsel bestaande uit dennehoutsaagsels, mieliemeel en sojameel gevestig. Die aantal eiers per wyfie was

tussen 100 en 250. ’n Nuwe verskynsel is vir die eerste keer vir *B. impatiens* waargeneem: die lê van eiers in ’n ketting. Die lengte van die eerste larvale stadium is tussen 1.2 en 2.5 mm, die van die tweede tussen 2.6 en 4.5 mm, die derde tussen 4.6 en 6.5 mm en die vierde tussen 6.5 en 7.2 mm. *Bradysia impatiens* se lewensiklus duur by 25°C ongeveer drie weke in ’n groeikamer. Daar is vasgestel dat *B. impatiens* as plaagspesie reeds goed gevestig is in Suid-Afrikaanse bedekteboerderystelsels.

Agt plaaslike Suid-Afrikaanse EPN-spesies en ’n uitheemse *S. feltiae* is getoets vir hul vermoë om die vierde larvale stadium van *B. impatiens* te beheer. Laboratorium toetse is uitgevoer om insekdodende aktiwiteit, dodelike dosis (LD) en die doeltreffendheid van die EPN by teen verskillende temperature vas te stel. Die resultate toon dat, van die verskillende laboratorium toetse wat uitgevoer is, vier plaaslike EPN-spesies nl. *Steinernema yirgalemense, Heterorhabditis noenieputensis*, *Heterorhabditis indica*, en *Heterorhabditis zealandica*, mortaliteit van meer as 80% behaal het teen 25°C en 30°C. *Heterorhabditis zealandica* het die laagste LD_{50} van 2.6 nematodes per insek larwe en ’n LD_{90} van 18.68 nematode per larwe gehad, terwyl *S. yirgalemense* en *H. indica* LD_{50} en LD_{90} van onderskeidelik 9; 64 en 9; 64 gehad het. *Steinernema yirgalemense*, *H. indica* en *H. zealandica* kon almal in *B. impatiens* se vierde larvale staduim reproduseer en ’n nuwe kohort IJ’s vorm. Nematodes wat betreklik groter is, soos in die geval van *S. jeffreyense*, *S. khoisanae* en *Steinernema* sp. (WS9), was nie daartoe in staat om *B. impatiens* se vierde larvale stadium te infekteer nie, wat aantoon dat die grootte van die EPNs hul vermoë om swammuggielarwes te infekteer, beïnvloed.

Die effek van verskillende konsentrasies van ’n plaaslike, *S. yirgalemense*, is getoets om vas te stel wat die potensiaal vir die bestryding van ’n natuurlike besmetting van *B. impatiens* in ’n kommersiële komkommerkweekhuis is. Daarbenewens is die effek van potmedia op nematodebeweging getoets om ’n kunsmatige populasie muggies te bestry. Die konsentrasies is gebaseer op die aanbevole konsentrasie *S. feltiae* (’n kommersiële produk) vir die bestryding van Sciaridae (5×10^5 IJ’s per m^2). Die verdubbeling van die aantal

nematodes in die aanbevole dosis het die aantal *B. impatiens* met 77% na 14 dae verminder en na 21 dae met 76%. Daar is egter waargeneem dat die muggiepopulasies baie vinnig tot hul oorspronklike vlakke herstel, wat die kort lewensiklus van die vlieg en die belangrikheid van voorafbehandeling en die herhaalde toediening van EPNs beklemtoon. Die drie verskillende tipes potmedia, bestaande uit dennehoutsaagsels, kokosklapperhaar en 'n mengsel van baalklapperhaar en vermikuliet, het almal 'n positiewe effek op die *S. yirgalemense*-infeksie gehad. Vir die muggielarwes wat by die media gevoeg is, is mortaliteit van > 75% behaal, soos aangetoon deur die aantal volwasse muggies wat op geel plakkaarte gevang is.

Die resultate van die huidige studie toon aan dat die plaaslik geïsoleerde EPN, d.w.s. *H. zealandica, S. yirgalemense* en *H. indica*, onder optimale toestande belowende potensiaal toon as biobestrydingsagense vir *B. impatiens* in te hou. Bykomende navorsing moet onderneem word na die impak van, onder andere, tydsberekening, konsentrasie en nematodetoedieningstegnieke, om verdere belangrike inligting in te win oor die veldtoepassings van nematodes. Verdere ondersoeke moet ook vasstel watter verskillende faktore die optimale veldprestasie van *S. yirgalemense* kan beïnvloed. Die betrokke faktore sou, onder andere, die voorafbehandeling van substrate, die gebruik van hoër konsentrasies EPN, opvolgtoedienings, die tydsberekening van EPN-toedienings, of voorafbehandeling met kadawertoediening teenoor die watersuspensiestoediening van die EPN kon insluit. Die toediening van nematodes moet egter nie as 'n alleenstaande behandeling gesien word nie, maar eerder as deel van 'n geïntegreerde bestuurstelsel vir plaagbestryding beskou word waar sanitasie as die belangrikste oorweging geag word.

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A review of *Bradysia* spp. (Diptera: Sciaridae) as pests in nursery and glasshouse crops, with special reference to biological control using entomopathogenic nematodes

Abstract

Fungus gnats, which are also commonly known as mushroom fly, nuisance flies, black fungus gnats, and dark-winged fungus gnats, are dark-coloured small flies that belong to the family Sciaridae. They are well distributed throughout nature, with most being beneficial, and acting as soil health indicators and as organic matter decomposers. However, a few species of the genus *Bradysia sensu latu* have been identified as pests of some important commercial crops, mainly in greenhouses, nursery beds, potted houseplants, and mushrooms. Although fungus gnats are principally mycophagous, they have also been described as opportunistic herbivores, making them potential plant pests. They are primarily a problem in high moisture environments. They cause direct damage on the plant through larval feeding and indirect damage through creation of entry points for plant pathogens. They also transfer fungal diseases between plants. The most important species that have been described as pests are *Bradysia coprophila* Lintner and *Bradysia impatiens* Johannsen. The latter species, which has a global distribution, has recently been identified in South African forest pine nursery beds. Control has mainly been achieved through the use of chemical insecticides, but also, to a considerable extent, the use of biocontrol agents such as entomopathogenic nematodes (EPNs). The use of EPNs is preferred, since fungus gnats are pests of such crops as mushrooms, or of houseplants that are in very close association with human beings. Control using EPNs has mainly been achieved through the use of *Steinernema feltiae*. This review aims to provide insights into the *Bradysia* spp. as pests in undercover crops, with special reference to biological control using EPNs.

Introduction

Individuals belonging to the family Sciaridae are known by many names. Most commonly, they are referred to as mushroom fly, nuisance flies, black fungus gnats and dark-winged fungus gnats, despite them all being flies, due to their possession both of a dark body and of dark wings (Mohrig & Menzel 2010; Mohrig *et al*. 2013), and to them being principally mycophagous (Nielsen 1997; Mohrig & Menzel 2010; Shin *et al.* 2015). Therefore, their existence, especially in agricultural plantations, has been associated with fungal mycelial growth around the crop environment, - that is the substrate and/or plant roots. This, in turn, relates to conditions of high moisture (Mansilla & Pastoriza 2001; Lee *et al*. 2010) and of decomposing organic matter.

Most sciarid larvae are decomposers, and they can be found in nature in different kinds of organic material and/or plant litter (Lee *et al*. 1999; Mohrig & Menzel 2010; Shin *et al.* 2015), such as decaying tree stumps and decomposing organic matter in the soil (Menzel *et al.* 2003; Barraclough & Londt 2008). Principally, they feed on fungi, algae and decomposing organic matter (Mansilla & Pastoriza 2001), but some mine the stems and leaves of herbaceous plants (Menzel *et al.* 2003; Barraclough & Londt 2008). Shin *et al.* (2013) recently classified the larval habitats of Sciaridae into three different types, depending on their feeding habits: living plants (larvae involved in mining plant roots, stems, or leaves); rotten wood (larvae involved in living on dead wood and/or under bark); and plant litter (larvae living in such soil fractions as humus or leaf litter). Being primarily decomposers of plant debris, most of the fungus gnat species are both harmless and beneficial, playing a significant role in organic matter mineralization, and with some working as bioindicators of soil health (Mohrig *et al.* 2013). Only less than 10 species have been known to specialize in feeding on living plant material (Mohrig *et al.* 2013).

Over time, the sciarid species that belong to the larval habitat classified as living plants, (Shin *et al.* 2013) have adapted to mining and feeding on living plant material, such

as roots, stems, leaves, flowers, or even the whole plant, as in the case of young, tender seedlings (Mohrig & Menzel 2010). Such adaptations have come to make the species involved potential pests (Mohrig & Menzel 2010) of many economically important crops, especially where favourable environmental conditions occur. The conditions concerned refer to the presence of a high amount of decomposing organic matter and to high humidity levels (Mansilla & Pastoriza 2001; Lee *et al.* 2010). Members of the group involved belong to the subfamily Megalosphyinae, genus *Phytosciara sensu latu*, and part of the genus *Bradysia sensu latu*. The members live in the tissues of living plants, being more associated with living plants than is any other Sciaridae group (Lee *et al.* 2010).

Studies indicate that sciarids have adapted to human environments, including gardens, greenhouses, mushroom cultures, and potted houseplants (Mohrig *et al.* 2013), where they tend to feed on decomposing matter including humus and/or soil (Shin *et al.* 2013) that can be found in the plant environment. They have also been observed to feed on animal excrement (Barraclough & Londt 2008). Fungus gnats have come to be regarded as generalist opportunistic herbivores that usually feed on organic matter and fungi, but that resort to eating roots and underground stem tissue upon depletion of their normal feed (Vaughan *et al.* 2011; Shin *et al.* 2013). However, Lee *et al.* (2010) mentions that the trend concerned could have been altered, as in the case of living plant-feeding larvae, “the hypothesized larval habitat of Sciaridae shifted during the course of evolution from dead plant to living plant” from an ancestral type of mixed dead plants. Thus, larvae can live on living plant parts that have been attacked by fungi (Lee *et al.* 2010).

Most such sciarid species have, in the past, received little or no attention, especially because of their misdiagnosis as fruit flies (Mohrig *et al.* 2013), with their effects also being underestimated. However, it is reasonable to assume that species that have adapted to feeding on living plant material should be potential crop pests. The species concerned have commonly been observed in greenhouses, nursery beds and flowerpots in residential homes

(Nielsen 1997; Mohrig *et al.* 2013), where their soil-dwelling stages inhabit the plant-growing media, feeding on and damaging plant roots and stems (Chandler *et al*. 2010).

The economic importance of *Bradysia* spp. in agriculture

Insect pests, in general, have a direct impact on agricultural food production, by chewing the leaves of crop plants, sucking out plant juices, boring within the roots, stems or leaves, and spreading plant pathogens (Popp & Hantos 2011). *Bradysia* spp. are no exception to the above. The financial losses that are incurred due to the depredations of pests are hard to calculate. However, insects are estimated to consume about 10 % of the gross domestic product (GDP) in large industrialized nations, and up to 25 % of the GDP in some developing countries (Popp & Hantos 2011). Chandler *et al*. (2010) estimate the economic losses that are experienced annually in the UK due to fly (fungus gnats and shore fly) larvae to be at least 5 % due to crop losses, to marketing problems, and to the contamination of herb and pot plant crops. The extent to which *Bradysia* spp. affect agricultural productivity could still be highly underestimated, considering their secondary effects of transmitting diseases, which are often overlooked. Of significant importance, is the fact that the adult flies swarm the workers, causing them discomfort (Schuhli *et al*. 2014), potentially resulting in low worker efficiency. For this reason, fungus gnat infestations in agricultural production should be taken seriously, and subsequent control measures should be undertaken to prevent further infestation, or to keep the fungus gnat populations under control.

The fungus gnat larvae, with their prominent chewing mouth parts, are able to feed on most plant parts, but especially on the young and developing radical system, the tender roots, and the root hairs, causing significant damage to the root system that is likely to result in notable plant root biomass loss (Cloyd & Zaborski 2004). The fungus gnat larvae have been shown to avoid the lignified part of the crop in some cases (Mansilla & Pastoriza 2001; Vaughan *et al*. 2011). Vaughan *et al*. (2011) showed that *Arabidopsis thaliana* (L.) Heynh.

(Brassicaceae) seedlings were notably more susceptible to damage by *Bradysia* spp. than were mature plants, resulting in overall decreased growth and survival rates for the affected seedlings. Such direct feeding and burrowing into plant roots (Vänninen 2003) and stems (Springer 1995; Mansilla & Pastoriza 2001) by the larvae, which is the main feeding stage of the species, causes physical damage to the plant, creating sites for plant pathogen entry and/or indirect damage through the transfer of soilborne diseases, especially such fungal diseases as *Pythium*, *Botrytis*, *Thievalopsis, Cylindrocladium,* and *Sclerotina* (Pundt 1999), *Fusarium oxysporum* Schlecht (Scarlett *et al*. 2014), *Phoma, Verticillium* (Ludwig & Oetting 2001), among others. The adult fly is another dispersal agent for the pathogens concerned, as it flies between plants (Pundt 1999).

The feeding of, and the damage that is caused by fungus gnat larvae in terms of the epidermis and cortex tissues (Springer 1995), which are significant parts of the root plant system, interfere with the plant´s ability to absorb water and nutrients. In addition, it limits the amount of carbohydrate storage and it weakens the structural support of the seedlings in particular, which, consequently, tend to fall over and are fed on by the fly larvae (Springer 1995). The symptoms that are presented by affected plants include wilting, loss of vigour, reduced vegetative development, and loss of leaves (Pundt 1999). The roots appear to be abraded, containing small brown lesions (Springer 1995). The effects concerned have the summative result of killing the whole plant off, especially in the case of such heavy infestations as have been reported by Springer (1995) and Mansilla & Pastoriza (2001), while the physical damage incurred tends to lead to weakness in the plants involved (Cloyd & Zaborski 2004). This problem not only reduces yield, but largely increases production costs per hectare in terms of pest control (Popp & Hantos 2011), thereby causing economic losses to the farmer. Fungus gnats are primarily a problem under conditions of excessive moisture (Chamber *et al*. 1993) that commonly occur during propagation, during which time cuttings and plugs are developing root systems (Jagdale *et al*. 2004).

Bradysia spp. form part of the Sciaridae belonging to the larval habitat category of living plants (Shin *et al.* 2013). Members of the genus have become major insect pests to many crops of agricultural importance globally, as has been reported by different researchers. The species have been reported as being pests of different crops, including greenhouse-grown ornamentals (Harris *et al*. 1995), eucalypt nursery beds (Mansilla & Pastoriza 2001), and vegetable gardens (Kim *et al.* 2004; Mohrig *et al.* 2013). They have also been reported in plug production, especially where cuttings are used (Cloyd & Zaborski 2004), such as in poinsettia cutting production (Vänninen 2003; Villanueva-Sánchez *et al.* 2013). Other crops that are similarly affected include legumes (Springer 1995), shiitake (mushrooms) (Shin *et al.* 2015), glasshouse-grown fuchsias (Gouge & Hague 1995b), and other kinds of mushrooms (Barraclough & Londt 2008). Some of the crop species concerned have been shown to be more susceptible than are others (Jagdale *et al.* 2004).

The most important members of the above-mentioned groups are *Bradysia coprophila* Lintner and *Bradysia impatiens* Johannsen (Cloyd 2008). However, other species have also been reported as crop pests, including the *Phytosciara procera* Winnertz, which is a pest of the ginseng plant (Lee *et al.* 2010). *Bradysia difformis* Frey, which is an identified pest in South African pine tree nursery beds (Hurley *et al*. 2007a,b), is reported to be an introduced species. The species, which is commonly found in flowerpots and greenhouses, is widely distributed throughout many parts of the world, including South Africa, Brazil, the Hawaiian Islands and Europe, with it having come to be a serious economic pest of greenhouse crops (Menzel *et al.* 2003). The same species has recently been identified in Mexico (Villanueva-Sánchez *et al.* 2013) in association with poinsettia plants.

For purposes of this study, it is important to note that the naming of the genus *Bradysia*, has been disputed over time. Mohrig *et al*. (2013) recently noted that *B. impatiens* has been referred to differently by numerous researchers. Synonyms of the term used for this species include *Bradysia (Chaetosciara) tristicula* var. *difformis* Frey, *Sciara* (*Lycoriella*) *hardyi* Shaw, *Bradysia paupera* Tuomikoski and *Bradysia agrestis* Sasakawa.

In South Africa, the effects of the above-mentioned species still go unnoticed and unreported by many affected farmers, as is evidenced by the existence of only a few studies having, as yet, been undertaken in relation to the identification of *Bradysia* spp., including in the Afrotropical region, in general (Kirk-Spriggs & Stuckenberg, 2009). For instance, no studies have yet been done as regards the distribution of the species, or as regards their economic importance as pests on the African continent.

Identification and classification

The higher classification of Sciaridae as a family is currently a contentious issue, with many sciarid genera still generally being poorly defined (Mohrig & Menzel 2010). The genus *Bradysia* has been reported as containing the most species of the family Sciaridae (Menzel *et al.* 2003; Mohrig *et al.* 2013; Shin *et al.* 2013), with about 400 species worldwide. The genus is considered to be the most disputed of all Sciaridae as concerns its taxonomy, because so many of its species are morphologically similar to one another (Mohrig & Menzel 2010; Heller *et al*. 2015). They lack updated keys (Mohrig *et al.* 2013), with their differentiation requiring the use of advanced techniques, including the extraction of the male hypopygia (Mohrig & Menzel 2010). Their identification, in many cases, requires deoxyribonucleic acid (DNA) barcoding (Shin *et al.* 2015), while their immature stages have hardly, as yet, been identified because of their lack of key morphological features (Shin *et al.* 2015). In addition to the above factors, their often cryptic mode of life (Menzel *et al.* 2003; Mohrig & Menzel 2010), as well as their small size, add to the challenges that face correct identification (Lee *et al.* 1999).

Identification

Despite their inconspicuous and uniform appearance, and their size being so small (Lee *et al.* 1999; Menzel *et al.* 2003; Mohrig & Menzel 2010) -that is almost microscopic, sciarids can be identified even under low microscope magnification, because of their simple, yet characteristic, wing venation (Mohrig *et al.* 2013). The only part of their wings comprising

a crossvein is the short radial sector (r-s) at their base (Mohrig *et al.* 2013) (Fig. 1). The crossvein (r-m) appears as a basal continuation of the radial sector (Barraclough & Londt 2008). A wing section showing the (r-m) and (r-s) is shown in Figure 1 below.

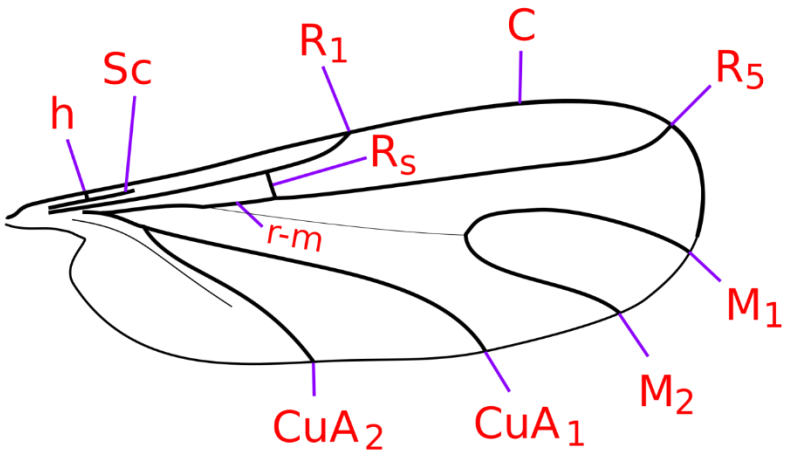

Figure 1.1 Photo showing the characteristic wing venation in Sciaridae (Tofilski, 2016).

Just as with all members of the Sciaridae, the adults of *Bradysia* spp. are dark-coloured small flies of uniform structure (Mohrig & Menzel 2010), which look like small mosquitoes, but which are dark/dark-brown in colour (Mansilla & Pastoriza 2001). The body size and the length of adult Sciaridae varies according to different researchers. Picker *et al*. (2004) described them as being small, with a 7-mm wingspan, and dark, being mostly about 2-3 mm long (Mohrig & Menzel 2010; Mohrig *et al.* 2013), while Menzel *et al.* (2003) mention that their body length is between 1-7 mm. Sciarids are rarely more than 10.0 mm in length (Mohrig & Menzel 2010). Mansilla & Pastoriza (2001) differentiated them in terms of the size of the different sexes, by demonstrating that the males of *B. difformis* are shorter (2.5 mm) in body length than are the females (3 mm). The subfamily Megalosphyinae, to which the genus *Bradysia* belongs, is differentiated by means of their possession of a comb-like row of bristles on the apex of the fore tibia (Mansilla & Pastoriza 2001). The feature in question could have come about as a synapomorphy of Megalosphyinae, evolved as an adaptation to living on herbaceous plants (Shin *et al.* 2013). However, the authors in question also recommend that further studies be undertaken to refine the interpretation of such a trend.

The females uniquely possess a long, bulged abdomen that ends with an ovipositor, whereas the males are distinguished by means of a narrower abdomen, ending with a distinct clasper. In addition to having a pale, transparent body, the larvae also possess a distinguishing shiny black, strongly chitinized head capsule (Lee *et al.* 1999).

Classification

As has previously been mentioned, the genus *Bradysia* belongs to the subfamily Megalosphyinae, which is differentiated by means of a strict comb-like row of bristles on the apex of the fore tibia (Mohrig & Menzel 2010; Shin *et al.* 2013). The classification of *Bradysia* spp. (Shin *et al.* 2015) has given rise to much controversy, with the sciaridae family only recently having been separated from the families Cecidimyiidae and Mycetophilidae (Picker *et al.* 2004; Barraclough & Londt 2008; Mohrig & Menzel 2010). The main differentiator is that the eyes of the sciaridae form a bridge above the antenna (Picker *et al.* 2004; Barraclough & Londt 2008; Mohrig & Menzel 2010). In a recent study by Shin *et al.* (2013), the genus *Bradysia sensu latu* was found to be polyphyletic, involving cladding with *Phytosciara*.

Life cycle

According to different authors, the duration of the life cycle of fungus gnats varies according to the temperature, being longer at lower ambient temperatures (Chandler *et al*. 2010). The same study reported that larval growth was very slow at 15°C, with development stopping at 10°C, and the optimum temperature for growth being 30°C. Some of the life cycle durations reported include: approximately 2 weeks (*B. impatiens*) (Kennedy 1973); 25 days at 20-25°C (Nielsen 1997); 3-4 weeks at 22-24°C (*B. paupera*) at about 70 % relative humidity (Mansilla & Pastoriza 2001); 26-28 days for *B. difformis* Frey at 25°C and 70 % RH (Villanueva-Sánchez *et al.* 2013); and 32-40 days for *P. procera* (Lee *et al.* 2010). The conclusion can, thus, be drawn that the duration of the life cycle of *Bradysia* spp. is highly temperature- dependent. The optimum temperature, as reported by Chandler *et al.* (2010), is

30°C. The lower duration of the life cycle at higher environmental temperatures, coupled with the ability of the female adult to lay many eggs, poses a threat, in terms of rapid population increases, for greenhouse farmers.

The life cycle of scarids is a complete metamorphosis, with the larva going through four instars. Mating usually occurs within the first 24 hours after adult emergence (Kennedy 1973), while oviposition tends to occur 1-2 days later.

The eggs: The eggs are oval-shaped, smooth, and shiny, of a clear, yellowish, semi-transparent colour (Mansilla & Pastoriza 2001), turning transparent towards the time of hatching, making it possible to observe the developing larvae (Villanueva-Sánchez *et al.* 2013). They are either laid in clusters, or individually (Kennedy 1973). The females normally lay their eggs on the surface of decomposing organic matter or soil. After a lapse of about 3-5 days, the larva emerges (Nielsen 1997; Lee *et al.* 2010).

The larvae: At approximately 3-5 days after oviposition, (depending on the prevailing temperature), the eggs hatch into larvae (Kennedy 1973). The colour of the larvae is translucent, and white to light yellow (Lee *et al.* 2010; Villanueva-Sánchez *et al.* 2013), turning yellow just before pupation. Mansilla & Pastoriza (2001) describe the larvae as thread-like, semi-transparent white, and having a black, shiny and strongly chitinized head capsule, which is a distinguishing feature (Cloyd 2008). The larva undergoes four developmental instars that are normally differentiated by means of their length, according to the following measurements: 0.4-0.6 mm (first instar), 0.6-1.25 mm (second instar), 1.25-2.5 mm (third instar) and 2.5-4.75 mm for the fourth instar (Mansilla & Pastoriza 2001). The larva develops into a pupa through the different instars that transpire within the space of about an average of 10 days as reported by different researchers. Nielsen (1997) reported12-14 days, while Mansilla & Pastoriza (2001) reported 9-13 days. Kennedy (1973) reported 13 days as the duration of the larval stage at 20°C and 9 days at 25°C. The first, third and fourth instars usually take a period of about 2-3 days, whereas the second instar takes about 3-4 days.

The larvae are not restricted to the ingestion of fungi, but they tend to feed on various kinds of plants and other rotting matter (Picker *et al.* 2004), while some species also tend to damage mushroom crops (Barraclough & Londt 2008). In preparation to pupation, the larva moves to the surface of the medium at a depth of about 2 cm (Mansilla & Pastoriza 2001; Chandler *et al.* 2010).

The pupae: The pupa is obtuse, with visible appendages that are very close to the body. Initially being white, it tends to develop at a later stage through a period of being yellow, to an ultimate golden brown. While its size is the same as that of the adult (Mansilla & Pastoriza 2001; Villanueva-Sánchez *et al.* 2013), its growth period lasts about 5 days (Nielsen 1997; Lee *et al.* 2010), depending on the prevailing temperature. However, the variation in duration is relatively insignificant, despite the temperature, with it being mostly about 3 days.

The adults: The adults are short-lived and rarely feed (Mohrig & Menzel 2010), even though some adults have been observed to feed on pollen or carbohydrate-rich sources (Mohrig & Menzel 2010). Although they have been reported to live for about 4-7 days, little is known about their habits (Mansilla & Pastoriza 2001; Barraclough & Londt 2008).

The adults lay a variable number of eggs, and earlier, Nielsen (1997) reported that they can lay as many as 1000 eggs on the media surface during the female's lifetime. However, recent studies indicate a much fewer eggs in comparison. Cloyd (2008) mentions that the females that he studied were each observed to deposit 100-200 eggs. Kim *et al.* (2004) report the production of about 121 eggs per adult for *B. agrestis*. Lee *et al.* (2010) reported that each female can lay between 100-200 eggs, in groups of 2-30. After the fungus gnats have become established in the environment, and because of the differences in, and the varying duration of, their life cycle, continuous and overlapping generations tend to exist within the same environment.

Diversity

Global occurrence

Sciarids, which are distributed globally in nearly all regions, tend to adapt very well to a variety of climates and fauna (Lee *et al.* 1999). Generally, fewer species of sciarids exist in an open landscape, as compared to other vegetation types, but the species are found in all terrestrial habitats consisting of forest and pasture (Mohrig & Menzel 2010). Such a wide distribution is probably because sciarid larvae have evolved during feeding on decomposing organic matter and fungi in forest litter. Megalosphyinae is the subfamily that adapts most successfully to environmental changes, as is indicated by the records of diverse larval habitats (including those that are characterized by the presence of dead plant material as well as living plants).

Most diverse groups of sciarids, such as *Bradysia*, are mainly phyto- and saprophagous. They can be collected from a variety of moist habitats, including from decomposing vegetation in forests, moorlands and greenhouses (Picker *et al.* 2004; Shin *et al.* 2013). Currently, over 5000 species of black fungus gnats live in such habitats worldwide (Mohrig *et al.* 2013).

Occurrence in South Africa

Sciaridae comprise the sixth least studied family of the Afrotropical dipterans (Barraclough & Londt 2008; Kirk-Spriggs & Stuckenberg, 2009). Even though the family has been very poorly studied so far, they are known to be prolific in wet forests and savannas. In 2004, there were about 26 described species known to be present in the Afrotropical region (Picker *et al.* 2004), while the following years saw the numbers increasing to 71 described species, representing 3 % of the predicted number of species, which is about 2000 (Pape *et al.* 2009). Southern African fauna are poorly known, with only five genera and ten species having so far been recorded (Barraclough & Londt 2008), with most of the species belonging

to the genus *Sciara*. Some of the species described for South Africa include *B. impatiens* and *Bradysia novalobata* Rudzinski (Rudzinski 1997; Hurley *et al.* 2007a).

In a study done in 2007 by Hurley *et al.* (2007a), as well as in their subsequent studies, the researchers concerned tried to establish the possible association of fungus gnats and other Diptera with pitch canker fungus in South African forestry nurseries (Hurley *et al.* 2007b). Their investigation took the form of sampling four major forestry nursery beds, with only *B. difformis* being identified in all the nurseries sampled. The assumption can, consequently, be made that not much diversity of *Bradysia* spp. as crop pests currently exists in South African agricultural areas. None the less, the studies involved have only sampled pine nursery beds. Therefore, there is still a need to sample other protected agricultural areas to find out the diversity of sciarids in South Africa in particular, but also throughout the whole of the Neotropical region, where insufficient information has, as yet, been discovered about such important dipterans.

Sources of introduction

Bradysia spp. are native to many parts of the world, although bagged soilless growing media / rooted plant plugs from wholesale distributors might be sources for the introduction of fungus gnats into commercial greenhouse facilities. Cloyd & Zaborski (2004) suggest that the pasteurization of even bagged soilless media might prove to be essential for the effective management of greenhouse populations of fungus gnats. However, this is not a viable means of response to already infested greenhouses.

Plugs of introduced vegetative material, as well as human activity (Mohrig *et al.* 2013), can be regarded as a source of *Bradysia* spp. in agrarian environments. Consequently, quarantine measures should be implemented to prevent their introduction through such a pathway.

Control measures

As with many other agricultural pests, the control of *Bradysia* spp. has been predominantly undertaken through the use of insecticides and biological control in rare cases, largely by means of entomopathogenic nematodes (EPNs) and parasitic mites. Many researchers have, however, argued that biological control measures can only be effective if they are supplemented with alternative plant protection management strategies (Chandler *et al.* 2010; Echegaray *et al*. 2015). The use of biological control measures can help to prevent problems that are associated with fungus gnats developing resistance to chemicals and to chemical residue in food crops, as well as providing a relatively sustainable approach to the control of fungus gnat pests. Different alternative strategies, including cultural, physical, chemical, and biological sanitation, as explained below, have already been both recommended and used for the control of fungus gnats.

Cultural and management practices

Fungus gnats have been shown to proliferate more vigorously under high moisture conditions of both growing media and the plant environment (Lee *et al.* 2010; Cloyd 2015). Restricting the water conditions of the growing medium to a minimum should prove to be an effective strategy for preventing the build-up of heavy infestations of fungus gnats (Bealmear 2010; Cloyd 2015).

Sanitation can be undertaken by means of eliminating the algae (Cloyd 2015) and decomposing debris that can act as breeding places for fungus gnats. Cementing the floors of undercover structures can serve as a way of preventing the algal growth on which fungus gnats feed. Cloyd (2015) also described other alternative cultural control strategies, such as the use of repellents and of physical barriers, both on the surface of growing media and around the crop environment, so as to prevent the fungus gnat adult from laying eggs on the growth medium concerned.

The culture media can also have an effect on fungus gnat proliferation. The selection of growing media can be used as a management tool, as the fungus gnat female adults tend to be more attracted to certain media than they are to others for purposes of egg laying (Cloyd *et al*. 2007). However, less attractive media have been indicated as possibly leading to more damage to the roots, since the larvae involved might, instead, resort to feeding on the roots (Cloyd *et al.* 2007). Yet, unfortunately, the currently preferred substrates, such as bark, wood fibre, coir, and composted green waste, seem to be more associated with fungus gnats than is the more conventional peat (Chandler *et al.* 2010). Pasteurization of soilless bagged media, prior to their introduction to an agrarian environment, could also serve as a control option, since they have been shown to be a source of introduction of fungus gnat eggs, especially in greenhouses (Cloyd & Zaborski 2004).

The adaptation of such gnats to human environments has enabled their dispersal by humans (Mohrig *et al.* 2013). Therefore, restricting entrance to farms for both humans and tools could is a necessary option for control of fungus gnats. By controlling such access, the entry of fungus gnats from neighbouring environments could be successfully limited.

The use of yellow sticky traps for capturing adults has also been used to control fungus gnats and to prevent them from laying eggs (Bealmear 2010). However, it is worthwhile noting that fungus gnat adults are weak fliers, requiring the placing of sticky traps near the growing media surface. Controversy does, however, exist regarding whether the number of insects that are trapped by the sticky traps is really an indication of the levels of infestation present (Harris *et al.* 1995; Vänninen 2003).

Another management option would be the monitoring of fungus gnat populations through the use of potato and carrot discs (Harris *et al*. 1995). These can be used to establish the existence of fungus gnat larvae in the growing-media so as to enable the taking of the correct timely control measures (Harris *et al*. 1995). For example, the preventive application of EPNs results in proper plant root development, which leads to the improved

quality and quantity of crop produce (Vänninen 2003). Also, fungus gnat adults can occasionally be monitored during watering, by means of gently shaking the plant, or by means of using sticky traps, or potato/carrot discs, to check for the larvae concerned (Bealmear 2010).

In terms of plug production, preventing the build-up of high levels of fungus gnat populations and the subsequent need for control might be costly. Vänninen (2003) and Kim *et al.* (2004) suggest the preventive application of nematodes as being the most practical management method, since it lowers the costs that are associated with direct treatment. The treatment concerned can include having to double the nominal EPN dosage, so as to achieve the actual required EPN densities in the plugs.

Even though there are currently no established thresholds for the application of control measures, the existence of a few fungus gnats might indicate the presence of an infestation, since a single female is capable of laying hundreds of fertile eggs. However, the maintenance of good root health could help to reduce the effects of fungus gnat larvae damage, since most of the larvae involved are likely to be concentrated in the top inch of soil (Mead & Fasulo 2014).

Chemical control

Generally, insect pest control has been conducted mainly through the use of chemical insecticides which, simultaneously, are the second most frequently used group of pesticides in the world (Tadeo *et al*. 2008). However, the abusive and careless use of such insecticides has brought about serious problems that are derived from a lack of specificity of the product and from its accumulation in the environment, which tends to lead to problems of pollution, as well as to the development of resistant populations (Neri *et al*. 2005). Inappropriate use of the chemicals concerned results into toxic effects and environmental contamination. This has led to an economic impact of about 8 billion dollars on non-targeted species, including humans, especially in developing countries (Aktar *et al*. 2009). Even though there are

chemical insecticides that are labelled for use against fungus gnats, it should be noted that no pesticides are registered for the control of fly pests on herbs (Chandler *et al*. 2010). Accordingly, more environmentally and humanly sustainable alternatives (Pal & McSpadden 2006) are called for, such as biological control (Tadeo *et al*. 2008), the use of resistant varieties and cultural control methods.

Insecticides have successfully been used for the control of fungus gnats, with some of the reported experiments including the effective use of flufenoxuron, diflubenzuron, deltametrina and azadiractina against *B. paupera* larvae (Mansilla & Pastoriza 2001), and organophosphate insecticide, diazinon (Harris *et al.* 1995), neonicotinoid insecticides, dinotefuran, imidacloprid, thiamethoxam, clothianidin against the larvae of *Bradysia* sp nr. *coprophila* (Lintner) (Cloyd & Dickinson 2006). Other successful experiments have been conducted using methicarb and chlorpyrifos for the control of *B. coprophila* (Ludwig & Oetting 2001), and diflubenzuron and chlorpyrifos for the control of *B. agrestis* (Kim *et al.* 2004). In addition, such insect growth regulators as pyriproxyfen (Cloyd *et al.* 2007), fenoxycarb, diflubenzuron, and azadirachtin have also been used (Ludwig & Oetting 2001).

Biological control of fungus gnats

Biological control in entomology entails the use of live organisms or of biological control agents, such as predatory insects, EPNs, or microbial pathogens, to suppress the populations of different insect pests (Pal & McSpadden 2006; Laznik *et al*. 2011). Many biological control agents have been reported to have been applied in attempts to control fungus gnats, some of which include, but which are not limited to, the following: predatory mites; soil bacterium; and entomopathogenic fungi (EPFs), EPNs and others.

Predatory mites

Predatory mites feed on both the eggs and the larvae of the fungus gnats. Their efficacy is sustained by their short life cycle of about 13 days, and by their ability to

manoeuvre their way through the soil profile (Jess & Bingham 2004; Bealmear 2010). Some of the predatory species of mites that have been used successfully for the control of sciaridae include the soil-dwelling predatory mites, *Hypoaspis miles* Berlese (Vänninen 2003; Jess & Bingham 2004), *Hypoaspis aculeifer* Canestrini (Jess & Bingham 2004), *Gaeolaelaps aculeifer* Canestrini (Grosman *et al*. 2011), *Macrocheles robustulus* Berlese (Grosman *et al.* 2011), which are also commercially available for the control of sciarids. However, it has been shown that EPNs can more easily survive in wet glasshouse conditions than can predatory mites, even though the latter might have a better dispersal ability within the substrate concerned (Vänninen 2003).

For example, Jess & Bingham (2004) used 700 mites of *H. aculeifer* and *H. miles* per m^2 for the control of sciarids, *Lycoriella ingenua* Dufour in mushroom compost, achieving a lower sciarid emergence in the treatments than was present in the controls. The same study reports that *H. aculeifer* provided a more comprehensive control of sciarids than did either *H. miles* or *S. feltiae*, due to its improved dispersal within the mushroom compost and casing substrates, and to its ability to attack larvae in different stages.

Soil bacterium

The soil bacterium, *Bacillus thuringiensis* Berliner subsp. *israelensis* (Bti) (Harris *et al.* 1995; Cloyd & Dickinson 2006), has been used for the control of fungus gnats. For example, Cloyd & Dickinson (2006) used *B. thuringiensis* subsp. *israelensis* (Bti), but the method did not show effectivity on the second and third instars of *Bradysia* sp. nr. *coprophila* Lintner. Such lack of effectivity suggests that the application of bacterium-based insecticides of this nature should occur prior to the build-up of overlapping generations of *Bradysia* sp. Other researchers have clarified that *B. thuringiensis* might not have the desired effect on the late instars of *Bradysia* sp., although it is effective with the first instar, since the initial larval stage has to consume relatively little of the bacterium for the latter to cause its death (Osborne *et al.* 1985).

Entomopathogenic fungi

A natural EPF, *Furia sciarae* Humber, that exists in relation to sciarids could be incorporated in the sciarid integrated pest management (IPM) control strategies, provided that its natural populations are protected and/or increased (Chandler *et al.* 2010). However, the culturing of this EPF is complicated, since the fungus can only be cultured using ballistospores. Such spores can be isolated from infected larvae, although, according to Chandler *et al.* (2010), they are normally contaminated. On testing the commonly used EPFs, *Beauveria bassiana* Bassi, *Metarhizium anisopliae* Metchnikoff *(*Sorokin*)* and *Lecanicillium lecanii* (Zimmerman), Chandler *et al.* (2010) found that their overall control efficacy against fungus gnats was low, with their effectivity being limited by the fact that they had to grow through the cuticle. Since the life cycle of *Bradysia* spp. is relatively short, the insect might moult before the spores of the EPF can achieve their desired effect. Pre-applications are thus recommended when using such EPFs. *Furia sciarae* (Olive) Humber has the differentiating characteristics that it can reproduce on insects and that it tends to spread rapidly throughout an insect population (Chandler *et al.* 2010).

Other biological control organisms, such as the Rove beetle, *Dalotia coriaria* Kraatz (Aleocharinae), have also been shown to effectively control fungus gnats in areas of protected agriculture (Echegaray *et al.* 2015). The aforementioned beetle is commercially available for the control of fungus gnats.

Biological control using EPNs

EPNs belonging to the family Steinernematidae and Heterorhabditidae are associated with mutualistic bacteria of the genera *Xenorhabdus* for Steinernematidae and *Photorhabdus* for Heterorhabditidae, which they carry in their intestines (Kaya *et al*. 1993; Kaya & Koppenhöfer 2004). The bacteria in question have the capacity to kill their insect host on entry (Dowds & Peters 2002), with them being, in fact, the true pathogens of the insect. They kill the insect by means of septicaemia (i.e. bacterial infection of the haemolymph), thus

facilitating the degradation of the related tissues, so that the nematode, together with its offspring, can feed on the tissue involved (Kaya *et al*. 1993; Vallet-Gely *et al*. 2008). Nobuyoshi (2002) describes the above method as consisting of a quick kill by means of the mutualistic associated bacteria. The lethal bacteria help to establish conditions that are suitable for the growth and reproduction of the nematode, as well as to inhibit further colonization of the cadaver by other microorganisms (Nobuyoshi 2002; Hazir *et al*. 2004), or, in some cases, by other nematodes of the same, and/or a different, species. The nematode-bacteria association is what permits the effectivity of EPNs (Shapiro-Ilan *et al*. 2010). The natural life cycle of EPNs and their symbiotic bacteria benefits both participants (Hazir *et al*. 2004). For example, the infective juveniles (IJs) of *Steinernema carpocapsae* Weiser contribute to the nutrition and growth of its bacterium *Xenorhabdus nematophila* Poinar and Thomas in the colonization site, while *X. nematophila*, in turn, contributes to *S. carpocapsae*'s reproductive fitness (Martens *et al*. 2005). EPNs, which can actively seek or ambush the host, are lethal to many important insect pests, but they are safe for both plants and animals (Shapiro-Ilan *et al*. 2010, 2012). They can be released in crop fields with insignificant effects on non-target insects, and they are regarded as being exceptionally safe to the environment (Kerry & Hominick 2002; Divya & Sanker 2009).

The IJ enters the larva through the latter's natural openings, including the mouth, the anus, and the spiracles. However, the IJs of *Heterorhabditis* species can penetrate through the cuticle directly into the insect's haemocoel, since they possess an anterior tooth that is specifically adapted for this purpose (Kaya *et al*. 1993; Nobuyoshi 2002). The nematodes feed, develop and reproduce within the insect cadaver. The IJs feed on the bacterial cells and decayed host tissues (Nobuyoshi 2002). Under ideal conditions, the IJs begin to exit from the cadaver from 7-15 days after infection, whereupon they begin to search for new hosts (Kaya *et al*. 1993).

EPNs belong to the order Rhabditida, with Steinernematidae and Heterorhabditidae being the two most commercialized families, which are used for the control of insect pests,

because they have presented remarkable results, as compared to what has been achieved with other families (Shapiro-Ilan *et al*. 2010). These two families, in particular, have emerged as excellent biocontrol agents of soil-dwelling insect pests (Grewal *et al*. 2005). They are now used to control a wide range of insect pests, including caterpillars, cutworms, crown borers, thrips, fungus gnats, and others that inhabit foliar, soil surface, cryptic and subterranean habitats, in relation to which they are lethal (Lacey & Georgis 2012). The use of EPNs has been facilitated even more by the discovery of multiple efficacious strains and by the desirability of reducing pesticide usage, making them useful in terms of both IPM and sustainable agriculture (Lacey & Georgis 2012).

Being living organisms, EPN efficacy is prone to both biotic factors and abiotic factors, including especially ultraviolet radiation, relative humidity, and temperature fluctuations. The results that are attained with the use of EPNs might also be unsatisfactory due to improper handling, transport and storage (Shapiro-Ilan *et al*. 2010). Selection of an EPN for the control of a particular pest insect is based on several factors, including the nematode's host range, its host-finding or foraging strategy, its tolerance of environmental factors, and its effects on survival and efficacy. The four most critical factors are moisture, temperature, pathogenicity for the targeted insect, and foraging strategy, within a favourable range of temperatures, adequate moisture, and the susceptibility of the host concerned (Kaya *et al*. 1993; Hazir *et al*. 2004).

EPNs can be applied in conjunction with chemical pesticides, soil amendments and fertilizers. IJs of EPNs have been found to be tolerant to short exposures (2-6 h) of most acaricides, fungicides, herbicides, and insecticides (Rovesti & Deseö, 1990; Rovesti *et al*. 2013; Laznik & Trdan 2014). They can, therefore, be applied simultaneously with many pesticides. This backs up their ability to be used in IPM practice. However, some studies have shown that some pesticides can reduce EPN viability and infectivity (Zimmerman & Cranshaw 1990; Alzugaray 1991; Krishnayya & Grewal 2002; Hazir *et al*. 2004; Rovesti *et al*. 2013; Echegaray *et al*. 2015).

EPNs are currently used in the biological control of certain insect pests, mainly because of their ease of mass production, their broad host range, and their safety for both mammals and the environment (Kerry & Hominick 2002). Such factors, as well as the advances that have been made in commercial production and formulation technology, have led to EPNs being used against a wide range of foliar pests (Brusselman *et al*. 2012; Schroer & Ehlers 2005; Beck *et al*. 2013). However, as with other biological control strategies, their successful use is influenced by a large number of complex interactions between the animals, plants and environment concerned (Kerry & Hominick 2002).

The use of EPNs in South Africa

Currently, a total of eight *Steinernema* and two *Heterorhabditis* are described as coming from South Africa (Malan & Hatting 2015), where soil surveys indicate that *H. bacteriophora* Poinar is the most abundant species. Some on the EPN isolates have already been tested for their potential to control different insect pests, such as codling moth, mealybugs, fruit flies, and the sugar cane stalk borer (De Waal *et al*. 2010; Malan *et al*. 2011; Ferreira & Malan 2014; Malan & Hatting 2015; Odendaal *et al.* 2016a; Odendaal *et al.* 2016b), with the studies concerned indicating that their field trials have had positive results. Thus far, biocontrol using EPNs in South Africa has not included the use of *S. feltiae*. Although *S. feltiae* has a global distribution, it has, to date, not been reported from the African continent, except for Algeria (Tarasco *et al*. 2009), where 13 strains of the nematode have been isolated. This emphasizes the need to test the South African local species for their potential to control insect pests in the region.

So far, special emphasis has been paid to *Heterorhabditis zealandica* Poinar, and to *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, since they have shown promising potential in terms of their virulence against insect pests in South Africa. In addition, they can be produced in great numbers with ease (Ferreira & Malan 2015;

Ferreira *et al*. 2015; Ferreira *et al*. 2016), as compared to the production rate that can be achieved with the other identified EPN species.

Although some of the isolated EPNs have already been used efficaciously in laboratory and limited field studies for the control of different insect pests in the region, none has yet been tested for the control of fungus gnats. The need exists to test the efficacy of different local South African isolates of EPNs on the control of fourth-instar larvae of *Bradysia* spp., which has been determined to be the most susceptible stage to *S. feltiae* (Gouge & Hague 1995a; Harris *et al.* 1995; Kim *et al.* 2004).

Previous research undertaken into the use of EPN to control *Bradysia* spp.

Preliminary evidence shows that EPN application is a potential method for fungus gnat management in plant plugs, nursery beds, greenhouses, and potted houseplants. However, numerous researchers recommend that fungus gnat control using EPNs be done early as a pre-treatment, in integration with other fungus gnat control options. In comparison with other control methods, EPNs are the most preferred strategy for the control of sciarids, since the latter are pests of protected crops when they are either close to consumption, as in the case of mushrooms, or where the application of pesticides might be considered undesirable, as in the case of houseplants or edible produce (Gouge & Hague 1995b).

The use of EPNs for the control of *Bradysia* spp. pests has had economic gains, especially in terms of plant plug production, where sciarid infestation would otherwise prevent the plugs from developing roots, which would result in plant loss. In a study done by Vänninen (2003) to control sciarids in Poinsettia plug production, treatments with nematodes resulted in an increase in income in two out of the four cases evaluated, with such an increase being attributed to a reduction in the number of unrooted cuttings. This was despite the difficulty that was experienced in estimating the economic feasibility of preventive nematode application, since the rooting success for poinsettia was still good even under conditions of high sciarid infestation.

The studies that have been done on the use of EPNs in the control of *Bradysia* spp., in different regions of the world, have, by and large, pointed to the use of *S. feltiae* for the control of fly larvae on different crops: greenhouse-grown poinsettias (Harris *et al.* 1995); jiffy plugs of poinsettia cuttings (Gouge & Hague 1995a; Vänninen 2003); glasshouse-grown fuchsias (Gouge & Hague 1995b); New Guinea impatiens; and greenhouse-grown poinsettia (Jagdale *et al*. 2004); mushroom compost (Jess & Bingham 2004) and production (Jess & Bingham 2004), and others. Only a few studies have been done on the use of other EPN species than *S. feltiae* to control fungus gnats. For example, Kim *et al.* (2004) used *S. carpocapsae Pocheon strain* on the third and fourth instars of *B. agrestis* in Korean greenhouses, achieving more than 80 % effectivity. However, Harris *et al.* (1995) observed no significant difference between *S. carpocarpsae* (All) and the control in their experiments to control *B. agrestis*.

Steinernema feltiae has been selected for its virulence against sciarids (Vänninen 2003), and for its ability to persist in the environment. The strain, which is commercially available for use against fungus gnats, is being used in Europe, to which area the nematode is native. Satisfactory results have been achieved with this species in relation to different fungus gnat species. A decrease of 92 % was found in the numbers of *B. paupera* adults emerging from potted compost (Gouge & Hague 1995b), and of 90 % in the efficient control of *B. paupera* larvae (Mansilla & Pastoriza 2001). Harris *et al.* (1995) found no statistical difference between the treatments using *S. feltiae* (SN) as compared to the treatments using the chemical insecticide, diazinon, suggesting that *S. feltiae* (SN) could prove to be a biorational alternative to conventional chemical insecticides.

However, the use of only *S. feltiae* as the ultimate EPN for fungus gnat control could pose challenges for use in other areas, especially where this species has not previously been isolated. Another limitation of the EPN species concerned is that *S. feltiae* has been shown to be less effective at higher temperatures (Gouge & Hague 1995a,b). For example, Gouge & Hague (1995a) tested different steinernematids and heterorhabditids to determine

their infectivity and mortality in relation to sciarid flies. In their study, *S. feltiae*, despite being found to be the most effective EPN for controlling sciarid species, was less effective at higher temperatures. Even under such conditions, the UK isolates of *S. feltiae* were more effective against UK sciarids than were the *S. feltiae* isolates from other European countries that were tested (Gouge & Hague 1995a). Jagdale *et al.* (2004), when indicating *S. feltiae* to work more effectively in a growth chamber (22°C), achieved a higher control efficiency of 73-80 % as compared to the mere 34-41 % that was obtained under greenhouse conditions, in which the temperatures were relatively high (above 25°C). Such findings show the necessity of using locally isolated species that might be better adapted to the prevailing environmental conditions.

In using EPNs for the control of sciarid larvae, the following factors require consideration, as has been highlighted by different researchers: the effect of EPNs on different stages of *Bradysia* spp.; the effect of potting media on EPN activity; and the concentration of EPNs.

The effect of EPNs on the different stages of Bradysia *spp.*

Different stages of *Bradysia* spp. have been shown to have contrasting susceptibility to EPNs. For example, while using the *S. carpocapsae* Pocheon strain, Kim *et al.* (2004) report the susceptibility of all *B. agrestis* stages, apart from the eggs. Kim *et al.* (2004) attribute such susceptibility to the lack of a large natural opening for the IJs to enter. In addition, they discuss that the chorion of the egg probably serves as an additional barrier to entry of EPNs. In other studies, first-instar larvae have shown minimal susceptibility, as compared with the older instar stages of *Bradysia* spp., which are reported to be more susceptible to EPNs (Gouge & Hague 1995a; Harris *et al.* 1995; Kim *et al.* 2004). The size of the sciarid larvae in comparison with the IJs is a debatable issue, with it having been reported that the IJs tend to enter the sciarid larvae through the mouth and anus (Gouge & Hague 1995b,c), since the spiracles are too small to permit their entry (Gouge & Hague 1995c; Hazir *et al.* 2004). While

using *S. feltiae* to control *L. ingenua* in mushroom compost, Jess and Bingham (2004) noted that the *S. feltiae* kept to a depth of 2-4 cm. Such a characteristic could even be an advantage to *S. feltiae*, since the *Bradysia* spp. larvae tend also to keep to the top centimetres of the substrate, especially towards the time of pupation, which coincides with the most susceptible stage of the sciarids (Jess & Bingham 2004). Thus, the control of fungus gnats using EPNs should target the adult instars of the larvae, which are more susceptible to their depredations.

The effect of potting media on EPN activity

The potting media has an effect on both nematode and fungus gnat larvae movement. Potting media is an important factor for the survival and infectivity of nematodes, as has been reported by different researchers. For example, Jagdale *et al.* (2004), found out that the efficacy of EPNs against the fungus gnat, *B. coprophila,* was higher in certain plant growth media than in others. It was also noted that certain potting media were more favourable to fungus gnat colonization than others. The same study argues that the presence of continuously decomposing media increases the water-holding capacity and reduces the porosity that increases the fungus gnat population, and it suppresses the action of several plant pathogenic organisms and plant-parasitic nematodes. It is, thus, important to use the right growing media when considering the use of EPNs as part of agrarian pest management strategy. The use of peat as a growth substrate is currently being challenged by the use of more sustainable alternatives (Chandler *et al*. 2010). Unfortunately, the currently advocated substrates, consisting mainly of composted green waste, bark, wood fibre, and coir, have been shown to be relatively closely associated with fly colonization (Chandler *et al.* 2010)

Concentration of EPNs

Different concentrations have been used during EPN applications for the control of *Bradysia* spp., which are normally determined as per m^2 of area under production (Gouge & Hague 1995a; Vänninen 2003).

For example, Harris *et al.* (1995) used 1.25 billion/ha *S. feltiae* for the control of *Bradysia agrestis* Sasakawa in jiffy plugs of poinsettia cuttings, in terms of which they achieved more than 80 % control. In using *S. feltiae* against *B. coprophila* on two New Guinea impatiens, Jagdale *et al.* (2004) employed 1.25×10^5 IJs/m^2 to achieve significant control of the fungus gnat, while having to use an application rate of 2.5 and 5×10^5 *S. feltiae* IJs/m^2 to achieve the same effect in terms of fungus gnat reduction on greenhouse-grown poinsettia. Mansilla & Pastoriza (2001) used *S. feltiae* for the control of *B. paupera*, achieving a mortality percentage of more than 90 %, while Jess & Bingham (2004) controlled sciarids in mushroom compost and in casing substrates using *S. feltiae* at a concentration of $3 \times 10^6/m^2$. Thus, different concentrations of IJs might be required to achieve the same efficacy in the case of various crops. Gouge & Hague (1995b) also noted that the nematodes were well distributed in the potted compost, and that they persisted in the compost over the 64-day experimental period. In their experiments, they also determined that adult sciarids can be infected by *S. feltiae*, and that they could, accordingly, help in the dispersal of the nematode.

Conclusions

Fungus gnats, and especially the species belonging to the genera *Phytosciara* s.l. and to part of the genus *Bradysia* s.l., are pests of greenhouse and ornamental plants, primarily being a problem under conditions of high moisture. Such gnats cause significant economic damage to undercover crop production through direct feeding on the plants concerned, through creating entry points for plant pathogens, or through the transfer of disease. The adoption of control measures, in the form of biological, cultural and chemical control, is

recommended in terms of an integrated system. However, care should be taken to prevent the build-up of large fungus gnat populations that would otherwise tend substantially to reduce crop yields. Constant monitoring for fungus gnats is also recommended. The biological control of plant-feeding *Bradysia* larvae in crop production using EPNs has been dominated by the use of *S. feltiae*. However, there is a need to test other EPNs so as to satisfy the demand in areas where the species has not yet been identified, or where it is less well adapted.

For South Africa, about 3 % of the predicted number of Sciarids is described. Currently, the identification and classification of fungus gnats is still a challenge, due to the lack of updated keys, especially in relation to the Afrotropical region, where only a few species have, so far, been identified.

Aim of the study

The main aim of this study is to investigate the potential use of EPNs for the control of *Bradysia* spp. under laboratory and greenhouse conditions. The objectives of the study are the following:

1. To identify and investigate the life cycle of *Bradysia* sp. occurring in South African glasshouse conditions.
2. To screen different EPN species for their potential use as a biological control agent against the larval stage of *Bradysia* spp.
3. To determine the effective application of EPNs under glasshouse conditions and the effect of different potting media.

The chapters of this study have been written as separate publishable papers, and, for this reason, some repetition, in the different chapters, has been unavoidable.

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Chapter 2

Identification, life cycle and artificial culture for fungus gnats, *Bradysia impatiens* (Diptera: Sciaridae)

Abstract

Bradysia spp. are important pests in mushroom cultures and in many other undercover crops. Their effects include direct physical damage of the roots, transfer of fungal diseases, and the creation of entry points for plant pathogens. *Bradysia* spp. are known to have a global distribution. Recently, they have been reported from South Africa in association with major tree nursery beds in the Mpumalanga and KwaZulu-Natal provinces (in 2007), and identified, as *Bradysia impatiens*, which is regarded as an introduced species. In this study, the same species was identified in association with other greenhouse crops in the Western Cape province, including herbs, cucumbers, mushrooms, blueberries, tomatoes, and chrysanthemums. The objective of the study was to identify a fungus gnat species causing problems in the greenhouses in the Western Cape, as well as to study its biology. Using molecular techniques and morphological observations, the species concerned was identified as *Bradysia impatiens*. Identified too, was *Lycoriella sativae*, which is reported for the first time, in South Africa and the Afrotropical region. A laboratory culture of *B. impatiens* was established using a 3:1:1 mixture of pine sawdust, cornmeal and soy meal. The number of eggs laid per single adult female was found to be between 100 and 250. A new phenomenon of egg laying, in the form of chains, was observed for the first time for *B. impatiens*. In length, the first instar larvae measured between 1.2 and 2.5 mm, the second instars between 2.6 and 4.5 mm, and the third instars between 4.6 and 6.5 mm, while the fourth instar larvae measured about 6.5 to 7.2 mm. The life cycle of *B. impatiens* lasted for approximately three weeks in a growth chamber, at 25°C. This study presents the first report of fungus gnats, *B. impatiens*, from tomatoes, herbs, and chrysanthemums, in South Africa.

Introduction

Fungus gnats have become major insect pests of greenhouse crops, nursery bed crops, house crops, and mushrooms. Chandler *et al*. (2010) estimated economic losses due to fungus gnats and shore flies to be accountable for at least 5% of crop losses, in terms of marketing problems and contamination, and in terms of herb and pot plant sales annually in the United Kingdom (UK). More so, the extent to which *Bradysia* spp. affect productivity could still be underestimated. Such a deduction can be made in view of their secondary effects, such as the transmitting of diseases and the creating of entry points for plant pathogens, which have, in the past, tended to be overlooked. In addition, fungus gnats cause discomfort to farm labourers (Schuhli *et al*. 2014), which may reduce worker productivity.

Bradysia spp. have been regarded as generalist opportunistic herbivores, usually feeding on organic matter and fungi, but turning to root and underground stem tissue upon depletion of their normal feed (Vaughan *et al*. 2011). However, Lee *et al.* (2010) clarified that this trend could have changed over time, with, for some species, “the hypothesized larval habitat of Sciaridae [having] shifted during the course of evolution from dead plants to living plants, thus larvae can live on living plant parts that have been attacked by fungi.” Fungus gnats are a problem primarily under conditions of excessive moisture, which commonly occur during propagation (Jagdale *et al*. 2004), at which time the cuttings and plugs are developing young root systems.

The sciarid’s larvae use their prominent chewing mouth parts to feed on most plant parts, especially in young and developing plant root systems, as well as on tender roots and root hairs. The sciarid pests have, in some cases, been shown to avoid the lignified part of the crop (Springer 1995; Mansilla & Pastoriza 2001; Vaughan *et al*. 2011). Their larval feeding results in significant physical damage to the roots, as well as to a decrease in the plant root biomass (Cloyd & Zaborski 2004). This kind of direct feeding and burrowing into

plant roots and stems (Springer 1995; Mansilla & Pastoriza 2001; Vänninen 2003) creates sites for potential plant pathogen entry. Indirect damage occurs through the transfer of soilborne diseases, especially fungal infections (Pundt 1999; Ludwig & Oetting 2001; Scarlett *et al*. 2014). Adult flies have also been reported to transfer fungal pathogens (Pundt 1999; Scarlett *et al*. 2014) when they fly from one plant to another.

The larval feeding on, and the resulting damage of, the epidermis and cortex tissues interferes with the ability of the plant to absorb water and nutrients. This feeding behaviour also limits the carbohydrate storage, changes the production of photohormones, and weakens the structural support of the plant, especially in the case of seedlings (Springer 1995). The symptoms that may be presented by the affected plants include wilting, loss of vigour, reduced vegetative development, and the loss of leaves (Pundt 1999). The roots generally appear to be abraded with small brown lesions (Springer 1995). The effects concerned may result in the death of the plant, especially in cases of heavy infestation, as have been reported by Springer (1995) and Mansilla & Pastoriza (2001), whereas the physical damage results in the weakening of the plants involved, which reduces their marketability (Cloyd & Zaborski 2004). The combined effects of pests not only reduce the resulting yield, but, to a large extent, increase the production costs per hectare (Popp & Hantos 2011).

Bradysia spp., as part of the Sciaridae belonging to the larval habitat classified as living plants by Shin *et al.* (2013) have become major insect pests to many crops of agricultural importance. The study mentioned, showed that the larvae classified under living plants are specialised in feeding on the different parts of living plants. The most important species involved are *Bradysia coprophila* Lintner and *Bradysia impatiens* Johannsen (Cloyd 2008), but other species have also been reported as crop pests. *Bradysia impatiens* Johannsen, is one of the sciarid pests identified in South African pine tree nursery beds, and is reported to be an introduced species (Hurley *et al*. 2007a,b). *Bradysia impatiens* Johannsen is synonymous with *Bradysia (Chaetosciara) tristicula* var. *difformis* Frey; *Sciara*

(*Lycoriella*) *hardyi* Shaw; *Bradysia paupera* Tuomikoski; *Bradysia agrestis* Sasakawa (Mohrig *et al*. 2013). The species, which is common in flowerpots and greenhouses, is widely distributed throughout Azerbaijan, Brazil, the Czech Republic, Finland, Germany, Great Britain, the Hawaiian Islands, Italy, Japan, Korea, Latvia, Mexico, Netherlands, Russia, South Africa, Spain, the USA, and Switzerland as a serious economic pest of greenhouse crops (Menzel *et al.* 2003; Shin *et al*. 2012; Villanueva-Sánchez *et al*. 2013).

The current study had the objective of collecting and identifying *Bradysia* spp. from eight targeted greenhouses in the Western Cape province of South Africa. The life cycle of *Bradysia impatiens* and their culture on artificial media, under laboratory conditions, was investigated.

Materials and methods

Insect collection

Adult fungus gnats were collected from four greenhouses which grow cucumbers, tomatoes, herbs, blueberries, mushrooms and chrysanthemums. All the farms are located in the Western Cape province of South Africa. For the commercial cucumber greenhouse, which was the principal target of this survey, infected cucumber stems, as well as the organic growing medium concerned, were collected. After the infected stems were cut into pieces, they were placed in closed plastic containers, lined with moist paper towels. The part of the stems that was cut, was that from the ground level up to about 20 cm high (Fig. 2.1), since the part of the dead plant in this region, in each case was found to be the most infected. The containers were kept moist in a growth chamber at 25°C, and the adult fungus gnats were allowed to emerge. For the rest of the farms, a similar procedure to the above-described was followed, but, in each case mentioned, only the growth substrate, including the organic growing media and/soil, was collected.

Identification of the Bradysia *species*

Both adults and the first-generation larvae from three of the farms were used in the molecular identification of the species by means of the COI gene. DNA was separately extracted from the whole larvae and adults, using the column-based QIAamp® DNA Micro extraction kit. The following Folmer primer set was used in the PCR reactions: LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'. The primer sets LCO1490 and HCO2198 are used to amplify a 658-bp fragment of the COI gene in a wide range of invertebrate taxa (Folmer *et al*., 1994). The resulting DNA sequences were edited using CLC Main Workbench (ver. 7.7.3), and blasted in BOLDSYSTEMS to determine the species identity. Specimens from each of the farms were morphologically verified by Hans-Georg Rudzinski (Entomo- graphisches Studio, Schwanewede, Germany) as *B. impatiens* while *Lycoriella sativae* was verified by Kai Heller (Germany).

Figure 2.1. An infected and dead cucumber stem, as a result of fungus gnat larval feeding. The cucumber is of the var. Litoral, grafted on a pumpkin stalk var. Ferro.

Bradysia *spp., rearing on artificial media*

The fungus gnats that were collected from the cucumber greenhouse were used to establish a culture in the laboratory. The method used to raise the fungus gnat culture was a combination of modified protocols employed by Cloyd & Dickinson (2006) and Lee *et al.* (2010). Greenhouse media, which consisted of partially composted, 3-mm pine wood chips (sawdust), were sterilised at 40°C overnight, and then allowed to cool to room temperature. Sterilising at the temperature concerned ensured the death of contaminating microorganisms, except for the fungal spores. The medium was then mixed with soy meal and cornmeal, in a ratio of 3:1:1, respectively. The mixture was used as the oviposition and growth medium throughout the experiments. Glass Petri dishes (100 × 20 mm) were lined with moist filter paper (Whatman No. 1, 90 mm), filled with moist growth media, covered, and left for 24 to 48 h in the growth chamber, so as to allow for fungal colonisation. The Petri dishes were then placed in a Perspex box, along with other Petri dishes containing adult fungus gnats. The new Petri dishes were kept moist, so as to attract the female adult fungus gnats for egg laying. After 24 h, the Petri dishes (now containing eggs), were removed, covered, and transferred to a growth chamber, where they were kept at a temperature of 25°C. The Petri dish covers were fitted with thin tissue paper to prevent the accumulation of condensed moisture on top. The Petri dishes were opened frequently for aeration, and a few drops of water were added to the mixture, daily, until the onset of pupation. Food, consisting of cornmeal and soy meal, was sprinkled on top of the dishes every second or third day, depending on the needs of the larvae population involved. On the onset of pupation, the amount of moisture provided was reduced. As soon as the adults emerged, the original Petri dish were transferred to the Perspex box, where it was left open for the adults to emerge, mate and fly to lay eggs in the new Petri dishes. The cycle was maintained for more than 10 generations. A new container was used for each generation, so as to prevent contamination of the later generations, especially in respect of mites. However, controlling all types of contamination, especially mites, in the medium was difficult, since the adult fungus gnats

were observed to carry mites on their bodies as they flew to new growth media, especially when the old growth medium was drying out. An extra effort to reduce the mite population was made through establishing cultures using fungus gnat larvae rinsed in distilled water. Even then, mites were still a constant problem in the growth medium. The Perspex box, containing the fly cultures, was kept at room temperature.

Life cycle of Bradysia *under laboratory conditions*

An oviposition and growth medium was prepared as described above, but, instead of using 3- mm wood chips, finer wood chips of about 500 micron were used. The fine wood chips were obtained by means of drying out the pine sawdust at 40°C overnight, and by blending the dry sawdust into fine pieces, using a commercial bar blender (stainless steel; HBB250SR). The resulting powder was then sieved through a 500-µm sieve. The smaller pine wood chips permitted easy observation and the counting of the eggs laid by each adult female fungus gnat. The smaller cell culture dishes (35 x 10 mm CELLSTAR®) that were used for the purpose were lined with 3.0 cm moist filter papers (Whatman®). Freshly emerged (neonate) adult fungus gnats (1 female and 2 male) were aspirated into the growth media, where they were allowed to mate and oviposit. After the death of the adult female, the eggs were counted using a light microscope, and oviposition behaviour was observed. It was important to count the eggs immediately, since, waiting any longer, fungi colonisation in the media would make it difficult to observe the egg laying patterns or count the eggs. The Petri dishes were then fitted with fine tissue paper on the Petri dish cover, and transferred to the growth chamber, so as to allow for the emergence and growth of the fungus gnat larvae. Individuals from each growth stage, consisting of the eggs, the different larval instars, the pupae, and the adults, were randomly removed from the medium, and their pictures taken using a Zeiss Stereo Discovery.V8 Microscope fitted with an Axiocam ERc 5s (n = 20). The ZEISS Labscope App for iPad was then used to measure the length and width of each selected individual.

Results

Insect collection

The greenhouses in the Western Cape province, from which the fungus gnats were collected during 2017, included four targeted greenhouses. The different crops produced, were herbs **1**(-33°51'21.4848" S, 018°59'16.4076" E), chrysanthemums and other cuttings **2**(-34°09'22.0176" S, 019°03'21.4776" E), herbs **3**(-33°49'43.2156" S, 018°55'03.0036" E), cucumbers, tomatoes **4**(-33°39'55.9728" S, 018°56'04.4268" E), blueberries **5**(33°48'38.6"S 18°55'23.7"E), blueberries **6**(33°49'41.7"S 18°55'03.6"E), mushrooms **7**(33°47'38.7"S 18°41'28.9"E) and mushrooms **8**(33°45'38.8"S 18°48'42.9"E). The localities from which *B. impatiens* is reported in South Africa, so far, are indicated in Figure 2.2 below, including one location for *L.sativae.*

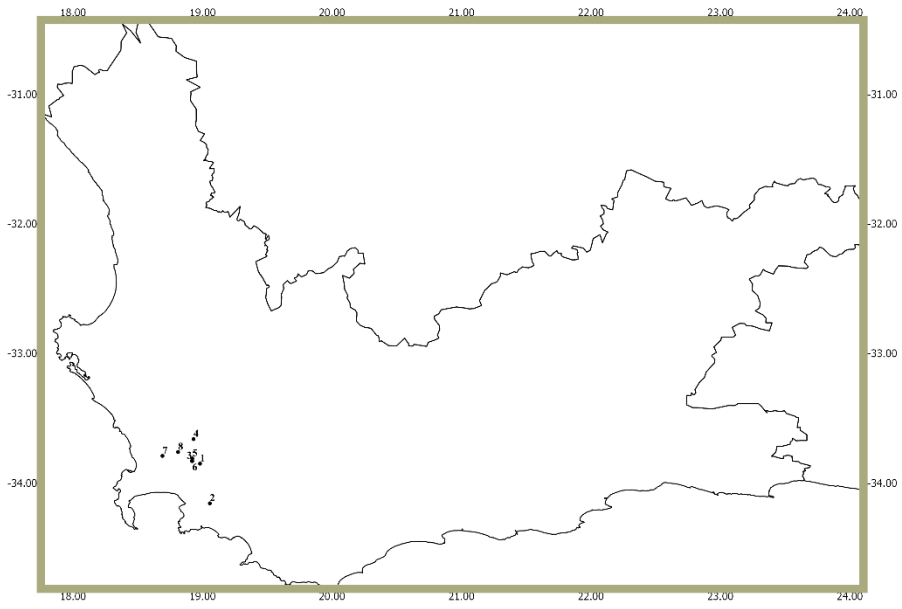

Figure 2.2. Map of the Western Cape province of South Africa showing localities from where fungus gnats were collected during the course of the current study (2017). The numbers correspond to different hosts as indicated in the text

Identification of Bradysia *species*

The resulting DNA sequences, when pasted into the BOLD program and GenBank, resulted in a 100% match with *Bradysia impatiens* Johannsen and *Lycoriella sativae* Johannsen as the fungus gnat species concerned.

The morphology of *B. impatiens* is synonymous with that of *Bradysia (Chaetosciara) tristicula* var. *difformis* Frey, *Sciara* (*Lycoriella*) *hardyi* Shaw, *Bradysia paupera* Tuomikoski, and *Bradysia agrestis* Sasakawa. *Braysia impatiens* is well studied, with some recent descriptions being given by Menzel *et al*. (2003) and Shin *et al*. (2012). The descriptions indicate that *B. impatiens* is easily recognised by the very short, compressed flagellomeres of the males concerned. The species involved is described as being the only species of the genus in which the males have shorter, or, at most, subequal, flagellomeres in relation to those of the females, requiring that it should not be confused with other *Bradysia* species with similar genitalia (fig. 2.3A). *Bradysia impatiens* belongs to the subfamily Megalosphyinae, which is differentiated by means of their possession of a strict comb-like row of bristles on the apex of the fore tibia (Mansilla & Pastoriza 2001; Mohrig & Menzel 2010; Shin *et al.* 2013). The females uniquely possess a long, bulged abdomen that ends with an ovipositor, whereas the males are distinguished by means of a narrower abdomen, ending with a distinct clasper, as is shown in Figure 2.3 C-D. Figure 2.3 B shows the simple, yet characteristic, wing venation of Sciarids. The only part of their wings comprising a cross vein is the short radial sector (r-s) at their base, whereas the cross vein (r-m) appears as a basal continuation of the radial sector (Mohrig *et al.* 2013).

Figure 2.3. A) Male genitalia of *Bradysia impatiens*, showing a comb-like row of bristles on the apex of the fore tibia, B) Wing of *Bradysia impatiens*, showing its characteristic wing venation, C) Female (broader) and male (narrower) of *B. impatiens*, D) Male adult.

Lycoriella sativae (fig. 2.4) is a well-studied species and its rediscription can be found in Menzel and Mohrig, (2000).

Figure 2.4. Male adult of *Lycoriella sativae* B) Male genitalia of *Lycoriella sativae*.

Rearing of Bradysia impatiens *on artificial media*

The culture medium used served as an ideal oviposition and growth medium for the fungus gnats. Up to 10 generations of fungus gnats were maintained on the same constitution of the culturing medium (Fig. 2.5), without any changes being noticed in the behaviour of the fungus gnat colony concerned.

Figure 2.5. A) Culturing medium, consisting of pine sawdust, soy meal and cornmeal, used to raise the colonies of *Bradysia impatiens*, B) Larvae of *B. impatiens.*

Life cycle of Bradysia impatiens *under laboratory conditions*

The adults: Recently emerged adults had a pale neonate body with wings that barely covered the abdomen. The wings recovered to full length in under 30 min after emergence. The emerged adults were very active. After a few hours, the adults had a dark-brown or dark body colour, and dark wings. Mating occurred within the first few hours after emergence. The body size of the adults was between 2 to 3 mm, with about 7 mm wingspan. Male adults were slightly smaller than were the females. The males hovered on top of the medium, with a bent abdomen, as they waited to mate with the emerging females. After mating, the

females lived for approximately 2 to 4 days, after which they laid eggs and normally died, either immediately after, or during, the process of egg laying. They lowered their abdomens into crevices to lay their eggs on top of the culturing medium, or just below the moist wooden chips. If the medium particles were small enough, as was the case with the 500-µm wood chips, the females lowered their abdomens into the medium, completely covering up the eggs.

The eggs: The egg-laying habits were variable among the female adults, which tended to lay eggs in groups of variable numbers, singly, while moving on top of the medium, or all at once, either in clusters, or in chains. The eggs were oval, with a light yellowish, shiny, semi-transparent colour as soon as they were laid, but which changed to colourless towards hatching (Fig. 2.6 A). Before hatching, the larvae, with their prominent black head, could be seen actively moving around and eating through the eggshell (Fig. 2.6 B). The eggs seemed to yellow more only under moisture. The eggs measured about 0.25 × 0.15 mm and seemed to yellow more, only under moisture stress. The number of eggs laid by the adult females, which was variable, and ranged between 100 and 250 (*n* = 20).

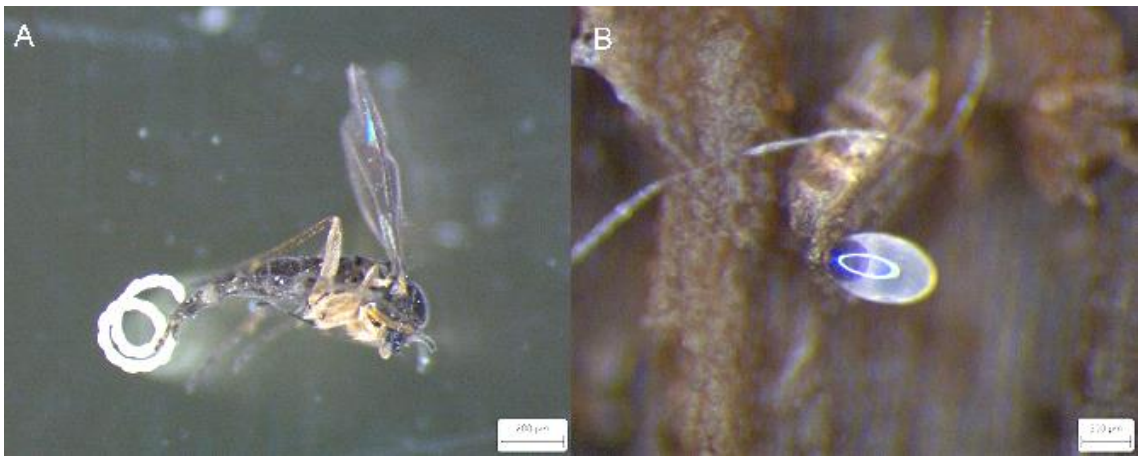

Figure 2.6. A) Egg laying in *Bradysia impatiens* in form of a chain, B) First instar larva about to hatch.

First instar larvae: After eating through the eggshell, the first instar became active immediately. Their body was white-transparent, while their head was shiny black, and chitinised (Fig. 2.7). The food and its colour could be seen through the semi-transparent

abdomen. The larvae quickly moved to the bottom of the medium, becoming active feeders. They were capable of surviving completely submerged in water, but were very susceptible to moisture stress. The first instar larvae measured about 1.2 to 2.5 mm in length, and from 0.2 to 0.3 mm in width (n = 20). The stage concerned lasted 2 days, after which they hatched into second instar larvae.

Second instar larvae: Except for their size, the second instar larvae were not behaviourally different from the first instars. They measured about 2.6 to 4.5 mm in length, and from 0.3 to 0.45 mm in width. The second instar larval stage lasted about 2 days, after which they hatched into third instar larvae.

Third instar larvae: The third instar larvae were more easily visible to the naked eye than were the earlier larval instars, and they could be seen eating through the medium. They were more slender than the fourth instar larvae. For the third instars and the previously mentioned instars, raw food substances could be seen inside their abdomen, thus, sometimes the larvae took on the colour of the food. They measured about 4.6 to 6.5 mm in length, and about 0.46 to 0.65 mm in diameter (n = 20).

Fourth instar larvae: The fourth instars were thicker than the previous instars, and contained almost no raw food substances, as they prepared to pupate. Thus, they appeared whiter than were the previously described instars. They measured about 6.5 to 7.2 mm in length, and ranged from 0.65 to 0.75 mm in diameter (n = 20).The fourth instar larvae migrated to the topmost layer of the medium, with their body continuously shortening, and, thereafter, pupated. The stage concerned lasted approximately 2 days. As the larvae moved, they left behind a characteristically slimy, shiny translucent gel. All the larvae were sensitive to the light, with them tending to hide under the medium when they were suddenly exposed to the microscope light.

Pupa: The pupae measured about 2 to 3 mm in length, and from 0.6 to 0.8 mm in diameter (n = 20). The pupae cocooned within the top layers of the medium, and, on

approximately the third day, the adult fungus gnats emerged. The pupae were yellowish-brown in colour, but they turned dark towards emergence as adults.

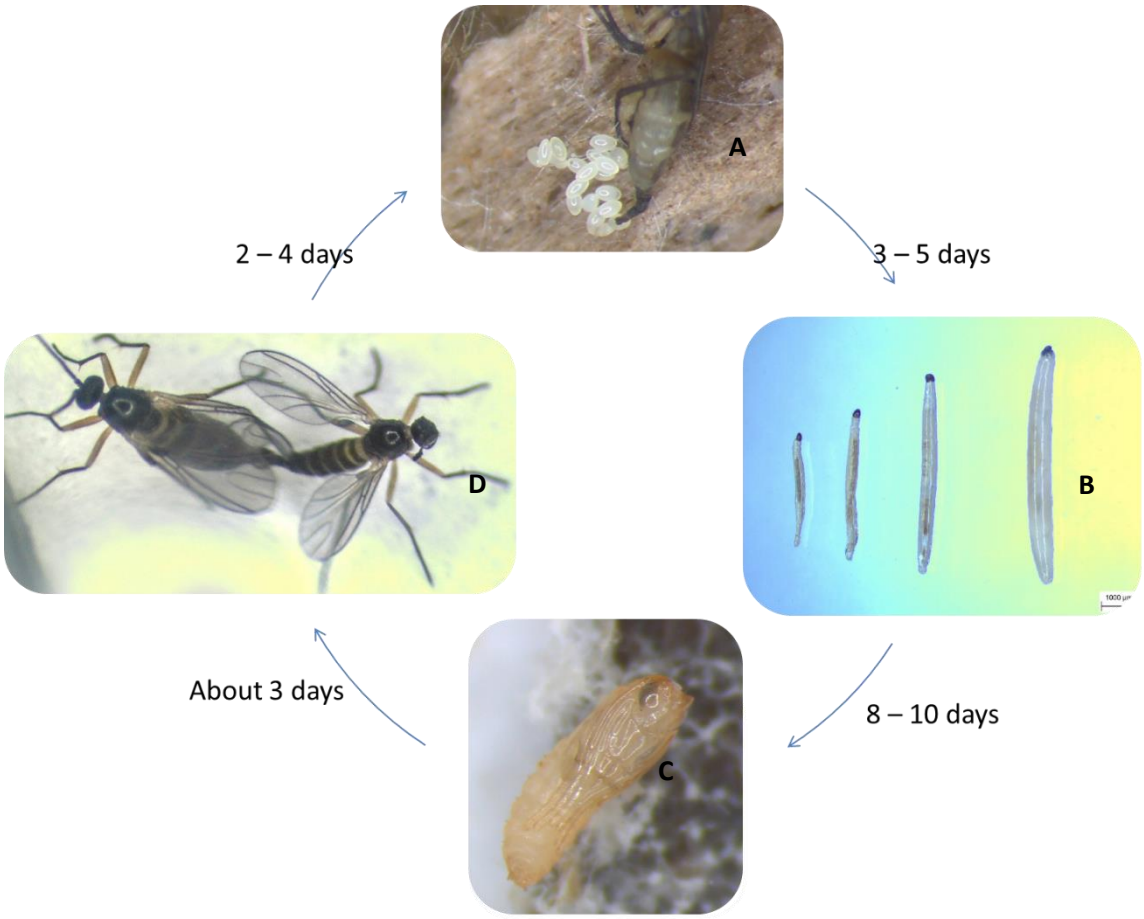

Figure 2.7. Observed life cycle of *Bradysia impatiens.* A) A batch of eggs laid by the *B. impatiens* adult, B) Four larval stages of *B. impatiens*, C) Pupa of *B. impatiens*, D) Male and female adults of *B. impatiens*.

Discussion

A recent study by Lee *et al*. (2010) clarified that a few fungus gnat species, belonging to the subfamily Megalosphyinae (genus *Phytosciara sensu latu*, and part of the genus *Bradysia sensu latu*), have more frequently had their larvae associated with living plants than has any other Sciaridae group. The larvae of these fungus gnat species are capable of mining into the roots, stems and leaves of living plants. For this matter, some species are important pests of those crops that are of global agricultural importance, especially in terms

of plants under cover, under which conditions the species are considered to be some of the most important pests worldwide. *Bradysia impatiens* (Mohrig *et al*. 2013), which is a major crop pest, has a global distribution, with it previously having been identified in South Africa in association with major forestry nurseries (Hurley *et al*. 2007a,b). In South Africa, the effects of the species concerned still tend to go unnoticed, and unreported, by many farmers. Only a few studies have been undertaken so far for purposes of identification, but none has yet been done on the biology, or management, of the species. At the time of the current study, *B. impatiens* had been identified as a serious pest on cucumber plants in a commercial greenhouse farm in the Western Cape province. *Bradysia impatiens* has also been identified in connection to other greenhouse crops, including tomatoes, mushrooms, blueberries, herbs, and chrysanthemums. Even though fungus gnats have previously been identified in South Africa in forestry nursery beds (Hurley *et al*. 2007a,b), the current study presents the first report of fungus gnats on any other crop, apart from pine nursery beds. This study also reports *L. sativae*, as a pest on mushroom, for the first time in the Afro tropical region. *Lycoriella sativae* is a well-known mushroom pest which had, up-to-date been identified to be well distributed in the Holarctic region and reported to have been distributed by man to Central America and the sub-Antarctic islands (Mohrig *et al*. 2012). Although another species, *Lycoriella ingenua* has been determined to be a more important pest species in mushrooms, *L. sativae* is known to be an agrarian species and the most abundant species of Sciaridae on fields (Mohrig *et al*. 2012, Mezel *et al*. 2013).

The biology of *B. impatiens* is known to differ according to different environmental conditions. The duration of the life cycle of sciarids has been regarded as distinctive by a variety of researchers. Such duration has been attributed mainly to the variable environmental temperatures, with less amount of time being taken to complete their life cycle at relatively high environmental temperatures. The optimum temperature for their growth has been determined to be 30°C (Chandler *et al*. 2010). In the current study, the duration of the life cycle of a laboratory culture of *B. impatiens* was found to be 2 to 3 weeks, at 25°C. On

observing the egg-laying habits of the species, apart from their commonly observed habits, a new phenomenon was detected, in terms of which the females were observed to lay their eggs in the form of a chain.

Different larval stage feeds have been described for the rearing of *Bradysia* spp. in the laboratory. Some of such feeds include the use of the fungal culture of *Pleurotus astreatus* grown on potato dextrose agar, PDA (Kim *et al.* 2004), a mixture of moist coconut coir dust, commercial rabbit food and brewer´s yeast, peat (Jagdale *et al*. 2004), potato agar (Mansilla & Pastoriza 2001), bacto-agar, and brewer´s yeast (Kennedy 1973). In all the combinations mentioned, fungi make up a basic element in the feed for fungus gnats. Upon death, the adult flies were observed to act as a primary source of fungi in the media as they deteriorate. This suggests that, even without a fungal culture, one could easily rely on the adult fungus gnats for the introduction of fungi into the new medium. The culture medium that was used in the current study, consisting of a 3:1:1 mixture of pine sawdust, soy meal and cornmeal, gave satisfactory results for more than 10 generations. Since some fungus gnat species have been shown to prefer laying eggs on cut planes, rather than on whole stems (Lee *et al.* 2010), the use of pine sawdust (blended) provided an ideal medium for egg laying.

During culturing, moisture was an important aspect in the growth of *B. impatiens* especially in the case of the larval instars, with the first instars in the current study being observed to be the most susceptible to moisture stress. As a consequence, water was added to the mixture more regularly during the larval stages, as they were the most susceptible to water stress. Aeration of the medium is equally important, with fungus gnats having been observed not to survive under anaerobic conditions. In the present experiment, the Petri dish cover was fitted with tissue paper to prevent the accumulation of condensed moisture on the upper lid, which, otherwise, was observed to be capable of creating anaerobic conditions if the condensed water created a water film on the edges of the Petri dish cover.

The fungus gnat species responsible for damage on a commercial cucumber farm was identified as *B. impatiens*. Under laboratory conditions, the life cycle of the species concerned was determined to be 2 to 3 weeks, at 25°C. This is the first report of fungus gnats laying eggs in the form of a chain, as well as the first report of *B. impatiens* as being a pest on any other crops than tree nursery beds in South Africa. The number of eggs laid by each single adult female, and their short life cycle, show that large numbers of fungus gnats can easily build up in a greenhouse, if control measures are not taken. *Bradysia impatiens* has been reported as an introduced species to South Africa, with a study by Hurley *et al*. (2007c) indicating that multiple strains of the species have been introduced into the country, possibly by the human importation of such contaminated material as substrates and ornamental plants, among others. Such an introduction emphasises the need for stringent restrictions to be placed on the importation of vegetative material, and for constant monitoring for such crop pests.

From the results of the current study, as well as on the basis of the findings of previous studies, *B. impatiens* can be concluded to be a well-established pest of protected crops in South Africa. However, the current study only targeted specific greenhouses to survey for fungus gnats. Such a limitation implies that more surveys are required to establish the existence of the species in other regions of South Africa, and the extent to which the species is a problem to South African growers. More important still is the need for studies regarding the sustainable management of the pests concerned, since they attack crops to which the application of chemicals is relatively inappropriate.

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Chapter 3

Potential use of local entomopathogenic nematode isolates to control *Bradysia impatiens* (Diptera: Sciaridae) under laboratory conditions

Abstract

Bradysia spp. are major pests of protected crops, to which they cause considerable economic losses. The use of entomopathogenic nematodes (EPNs) for the control of *Bradysia* spp. has proved to be relatively advantageous, since sciarids are mostly pests of protected crops that are either close to harvest, or for which, else, the application of pesticides is unsuitable. In this study, eight South African local species and a foreign *S. feltiae* were tested for their ability to kill *Bradysia impatiens* larvae. Bioassay protocols were performed to determine the insecticidal activity, the lethal dose, and the efficacy of the EPNs at different temperatures. The results showed four local EPN species, *S. yirgalemense, H. noenieputensis*, *H. indica* and *H. zealandica*, achieving higher than 80 % mortality, at 25 °C and 30 °C, from the different bioassays performed. *Heterorhabditis zealandica* had the lowest LD_{50} of 2.6 infective juveniles (IJs) per larva and LD_{90} of 18.68 IJs/larva, while *S. yirgalemense* and *H. indica* had LD_{50} and LD_{90} of 8.98; 64.16 and 8.98; 64.13 respectively. *Steinernema yirgalemense, H. indica* and *H. zealandica* were all able to reproduce inside the fourth-instar larvae of *B. impatiens*, and to produce IJs. The relatively bigger nematodes *S. jeffreyense*, *S. khoisanae*, and *Steinernema* sp. were unable to infect the fourth-instar larvae of *B. impatiens*, which showed that the size of the EPNs affected their ability to infect fungus gnat larvae. In this study, the high potential for the use of locally isolated EPNs for the control of fungus gnats in South Africa was demonstrated.

Introduction

Fungus gnats are major insect pests of undercover crops (Vaughan *et al*. 2011; Jagdale *et al*. 2004). The species concerned belong to the subfamily Megalosphyinae, the genus *Phytosciara sensu latu*, and part of the genus *Bradysia sensu latu* and form part of the Sciaridae belonging to the larval habitat category of living plants (Shin *et al.* 2013). Members of the genera are capable of chewing and mining into living plant parts, thus resulting in economic damages for the farmer. Chandler *et al*. (2010) estimated such losses to be at least 5 % (fungus gnat and shore fly larvae) in the United Kingdom. Apart from physical damage, fungus gnats transmit and disperse fungal diseases between plants (Pundt 1999; Ludwig & Oetting 2001; Scarlett *et al*. 2014). The adult flies also act as a nuisance, as they tend to swarm farm workers, causing them discomfort (Schuhli *et al*. 2014).

With their prominent chewing mouth parts, the fungus gnat larvae are able to feed on most plant parts, especially on young and developing radical systems, tender roots and root hairs. This causes significant damage to the root system, and it is likely to result in notable plant root biomass loss (Springer 1995; Cloyd & Zaborski 2004). The feeding of, and the damage that is caused by, fungus gnat larvae to the epidermis and cortex tissues (Springer 1995) interfere with the plant´s ability to absorb water and nutrients. This limits the carbohydrate storage, and it weakens the structural support of the plant (Springer 1995). The symptoms that are presented by affected plants include wilting, loss of vigour, reduced vegetative development, abraded roots, and the loss of leaves (Springer 1995; Pundt 1999), with eventual plant death, as is the case in the presence of heavy fungus gnat infestations (Springer 1995; Mansilla & Pastoriza 2001; Cloyd & Zaborski 2004). The effect on the farmer is reduction in plant yield and increased costs of production in terms of pest management (Popp & Hantos 2011).

The most important members of the above-mentioned groups are *Bradysia coprophila* Lintner and *Bradysia impatiens* Johannsen (Cloyd 2008). Other species have also been reported as crop pests, including the *Phytosciara procera* Winnertz, which is a pest of the ginseng plant (Lee *et al.* 2010). *Bradysia difformis* Frey, which has been identified in South African tree nursery beds (Hurley *et al*. 2007a,b), is reported as an introduced species. The species, which is commonly found in flowerpots and greenhouses, is reported to be widely distributed throughout many parts of the world, including South Africa, Brazil, the Hawaiian Islands, and Europe (Menzel *et al.* 2003). The same species has recently been identified in Mexico (Villanueva-Sánchez *et al.* 2013), in association with poinsettia plants.

The use of chemical pesticides has dominated the control strategies for fungus gnats. However, with regard to the biological control of fungus gnats, the use of entomopathogenic nematodes (EPNs) has been a preferred option. This is partly because sciarids are pests of protected crops that are either close to consumption, as is the case with vegetables and mushrooms, or else where application of pesticides might be considered to be undesirable, as is the case with houseplants (Gouge & Hague 1995b). Preliminary evidence shows that EPN application is a potential method for managing fungus gnats. Researchers advise that fungus gnat control using EPNs should be done early, and in integration with other fungus gnat control measures (Cloyd 2015). The use of EPNs for the control of *Bradysia* spp. results in economic gains, especially in plant plug production, in terms of which sciarid infestation would otherwise prevent the plugs from developing roots (Vänninen 2003).

EPNs belong to the order Rhabditida, with Steinernematidae and Heterorhabditidae being the two most commercialised families (Shapiro-Ilan *et al*. 2010). They are used for the control of insect pests, because they have presented remarkable results as compared to other families (Shapiro-Ilan *et al*. 2010). Particularly, the two families have emerged as excellent biocontrol agents of soil-dwelling insect pests (Grewal *et al*. 2005), and they are now used to control a wide range of insect pests, from foliar, soil surface, cryptic, and subterranean habitats (Lacey & Georgis 2012). Since fungus gnats spend most of their life

cycle stages in the soil/substrate, it gives a window of opportunity for the application of EPNs. The use of EPNs has been made even more possible through the discovery of multiple efficacious strains and the desirability to reduce pesticide usage (Lacey & Georgis 2012). Since EPNs have no known secondary effect on non-target organisms, this makes them suitable for integrated pest management and sustainable agriculture.

Most of the studies done on the use of EPNs in terms of the control of *Bradysia* spp. point to the use of *Steinernema feltiae* Filipjev, Wouts, Mráček, Gerdin & Bedding. Examples where *S. feltiae* have been used include: greenhouse-grown poinsettias (Harris *et al*. 1995), jiffy plugs of poinsettia cuttings (Gouge & Hague 1995a; Vänninen 2003), glasshouse-grown fuchsias (Gouge & Hague 1995b), poinsettia (Jagdale *et al*. 2004), New Guinea impatiens, mushroom compost (Jess & Bingham 2004), and mushrooms (Jess & Bingham 2004). Only a few studies have been done on the use of any other EPN species to control fungus gnats apart from *S. feltiae;* for example, Kim *et al.* (2004) used *Steinernema carpocapsae* (Weiser 1955) Wouts, Mráček, Gerdin & Bedding, a Korean isolate, on the third- and fourth-instars of *Bradysia agrestis* Sasakawa in Korean greenhouses, achieving more than 80 % efficacy. Satisfactory results have been achieved using *S. feltiae* to control sciarids. For example, 80 % control of *B. coprophila* larvae was achieved using 2.5×10^5 of *S. feltiae*/m^2 (Harris *et al.* 1995), a 92 % decrease in the numbers of *Bradysia paupera* Tuomikoski adults emerging from the potted compost was achieved with the use of 7.8×10^5 nematodes/m^2 by Gouge & Hague (1995b), while Mansilla & Pastoriza (2001) achieved 90 % control of *B. paupera* larvae.

However, the use of only *S. feltiae* as the ultimate EPN for fungus gnat control poses challenges for use in certain countries, especially where this species has not previously been isolated. *Steinernema feltiae* has also been shown to have biological limitations, especially at relatively high temperatures (Gouge & Hague 1995a,b; Jagdale *et al.* 2004). Although having worldwide distribution, on the African continent, *S. feltiae* has only been isolated from Algeria (Tarasco *et al*. 2009). In South Africa, such a situation has created a

need to test locally isolated EPN species that may be better adapted to the local environment. This is especially so because the introduction of foreign strains into a country should be properly controlled, since, otherwise, it might have adverse environmental effects (Alpert 2006).

The objective of the current study was to screen different isolates of EPNs from South Africa for their potential to control *B. impatiens*, both under optimum conditions. Laboratory bioassays were performed on the fourth-instar larvae of *B. impatiens.* Temperature trials were done using the resulting four most efficient local EPN isolates, and their lethal dosages were determined. The present study provided the first biological control study for fungus gnats, using local EPN isolates from South Africa.

Materials and methods

Source of nematodes

Nine EPN species, including eight isolates from South Africa, as well as an imported isolate of *S. feltiae,* were tested for their pathogenicity against fourth-instar larvae of *B. impatiens*. *Steinernema feltiae* was obtained from e-nema, Schwentinental, Germany, with the local species being obtained from the EPN collection of the nematology laboratory at the Department of Conservation Ecology and Entomology, Stellenbosch University. The EPN species used in the study are indicated in Table 3.1.

Table 3.1. Different *Steinernema* and *Heterorhabditis* isolates, with their associated host plants, origin, Genbank accession number, and the length and width of the infective juveniles (IJ) in micron (µm), used in the study.

Species	Isolate	Associated host plant	Origin (town/ province)	Genbank accession number (ITS region)	Length of IJ (µm)	Width of IJ (µm)
*S. jeffreyense**	J194	Guava tree	Jeffrey's Bay, Eastern Cape	KC897093	924 (784-1043)	35 (23–43)
*S. khoisanae**	SF87	Apple orchard	Villiersdorp, Western Cape	DQ314287	1062 (994-1159)	31(27-34)
S. yirgalemense	157-C	Citrus orchard	Friedenheim, Mpumalanga	EU625295	635 (548-693)	29 (24-33)
Steinernema sp.*	WS9	Litchi orchard	Nelspruit, Mpumalanga	KP325086	1054 (953-1146)	35 (29-41)
S. feltiae	e-nema	-	Germany	-	879 (766-928)	29 (26-32)
H. bacteriophora	SF351	Grapevine	Wellington, Western Cape	-	588 (512-671)	23 (18-31)
*H. noenieputensis**	SF669	Fig tree	Noenieput, Northern Cape	JN620538	528 (484-563)	21 (19–23)
H. zealandica	SF41	Natural vegetation	Patensie, Eastern Cape	-	685 (570-740)	27 (22-30)
H. indica	SGS	Grapevine	Bonnievale, Western Cape	GQ377411	528 (479-573)	20 (19-22)

*Type specimen from South Africa.

Freshly harvested EPNs were obtained through culturing in the laboratory, using mealworms, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), or last-instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae), in growth chambers at 25 °C. White´s traps (White 1927) were used to harvest the EPNs, within one week after emergence. Harvested IJs were then stored in distilled water at 12 °C, in horizontally placed culture flasks. The stored EPNs were shaken periodically for ventilation, and the EPNs were used within three weeks after harvest to ensure nematode viability.

Source of insects

A culture of *B. impatiens* was raised and maintained in the laboratory using a 3:1:1 ratio of partially decomposed pine wood sawdust, soy meal and corn meal, respectively. Only the fourth-instar larvae of *B. impatiens* were used in the experiments. The larvae of *T. molitor* were initially obtained from a pet shop, and afterwards cultured in vented plastic containers on fine bran and carrots, while the larvae of *G. mellonella* were raised on an artificial diet in the laboratory, in a growth chamber kept at 25 °C (Van Zyl & Malan 2015).

Screening of different EPN species

Nine EPN species (Table 3.1) were tested for their potential to kill fourth-instar larvae of *B. impatiens,* using twenty-four-well bioassay plates. Each bioassay plate contained 10 larvae, placed alternately in the wells. Each of the wells was inoculated with 100 IJs in 50 µl of distilled water, while the controls received water only. The lid of each well was fitted with two thin paper towels to prevent the escape of *B. impatiens* larvae. Five 24-well-plates, with 10 wells in each (n = 50), were used for each nematode species. The plates were placed in a plastic container lined with wet paper towels (100 % moisture), and kept in a growth chamber at 25 °C for a period of 48 h. Dead larvae were removed after 48 h, and then rinsed with distilled water, and dead larvae belonging to the same bioassay plate were placed in a small cell culture dish (35 * 10 mm) CELL STAR®, fitted with a piece of filter paper. These

were again placed in 100 % humidity plastic containers and transferred to a growth chamber at 25 °C for a further 48 h. This procedure allowed for EPN growth, as well as for easy observation and counting. Mortality was confirmed by means of visual observation of the nematodes in the cadavers by means of dissecting the dead larvae with the aid of a light microscope, and the number of nematodes present was recorded. The experiment was repeated for each nematode species on a different test date, using a fresh batch of nematodes.

Temperature trials

Five species, consisting of *S. yirgalemense*, *H. indica*, *H. zealandica, H. noenieputensis,* and *S. feltiae*, were selected from the screening trial and tested at 13 °C and 30 °C. Similar screening procedures were followed. At 13 °C, no mortality was recorded after 48 h for the local species. Thus, all the larvae present were removed, and rinsed in distilled water. The larvae belonging to the same well-plate were then placed in a small cell culture dish (35 * 10 mm) CELL STAR®, fitted with a piece of filter paper. These were again placed in 100 % humidity plastic containers, and transferred to the growth chamber at 25 °C for another 48 h, to determine whether penetration had occurred at 13 °C. The extent of mortality caused by visual nematode infection was recorded. The experiment was repeated on a different test date with a different batch of nematodes.

LD_{50} and LD_{90} tests

The three most effective EPN species, *H. zealandica*, *H. indica* and *S. yirgalemense*, from the previous experiments were tested to determine their lethal concentrations. Different EPN concentrations of 0, 5, 10, 20, 40, 80, and 160 IJs/larva were used. Twenty-four-well plates were used as the test arena, with each containing 10 fourth-instar larvae of *B. impatiens* that were placed alternately in wells lined with filter paper. In common with the set-up in the above experiments, five 24-well plates (n = 50) were used for each EPN concentration for each species concerned. Pieces of filter paper were placed into 10 of the

wells, to which the larvae were added, followed by the corresponding concentration of IJs in 50 µl of distilled water. For the control plates, only water was used. The plates of each treatment were placed in a plastic container lined with moist tissue paper (100 % moisture), and kept in a growth chamber at 25 °C for 48 h. Thereafter, the percentage mortality of larvae was determined and recorded.

Temporal development of the EPNs in B. impatiens

Fourth-instar larvae of *B. impatiens* were inoculated with *H. zealandica*, *H. indica* and *S. yirgalemense* to determine the temporal growth of the EPNs inside the larvae. Twenty 24-well plates (*n* = 200) were used for each EPN species. After placing pieces of filter paper alternately into ten wells of the 24-well-plate, the larvae were added, followed by 100 IJs of the EPN species in 50 µl of distilled water. The plates concerned that were treated with the different species were then placed in plastic containers lined with moist tissue paper (100 % moisture), and kept in a growth chamber at 25 °C. After 24 h had elapsed, the dead larvae were removed, and rinsed with distilled water. These cadavers were then kept in small cell culture dishes to continue observing the temporal development of the EPNs. For each species, 10 small cell culture dishes (35 * 10 mm) CELL STAR® were lined with wet filter paper. Ten larvae were placed in each small cell culture dish, and sealed with PARAFILM®. The 10 small cell culture dishes belonging to the same EPN species, after being placed in plastic containers lined with wet paper tissue, were transferred back to the growth chamber at 25 °C. After every 24 h had elapsed, a random petri dish was removed, the contents quickly observed under a light microscope, and then transferred to a freezer kept at 4 °C to terminate nematode development. After about 6 days, when the IJs were observed to begin emerging from the larvae, the experiment was terminated, and the rest of the petri dishes were transferred to White traps to enable the harvesting of the emerging IJs. Seven days after emergence, the number of IJs collected in the water was counted by taking out five 10-µl samples, and counting the number of IJs under a light microscope.

Statistical analysis

Statistical analyses were conducted using STATISTICA 13.2 software (StatSoft. Inc. 2016) for EPN screening and temperature trials, while a probit analysis (Finney 1952) was conducted using Polo PC (LeOra Software 1987) to determine the lethal dosages concerned. In the absence of significant differences between the test dates and the treatments involved, the data concerned were pooled and analysed using ANOVA, and a post-hoc comparison of means was undertaken, using Bonferroni's method. A bootstrap multi-comparison was performed (Efron & Tibshirani 1993), in case the residuals were not normally distributed. The data were expressed as weighted means ± standard error for EPN screening and temperature trials, and least square means ± standard error for the lethal dosage trials.

Results

Screening of different EPN species

The treatment and test date were analysed as the main effects, using a two-way ANOVA, and, as no significant differences were obtained, the results from the two test dates were pooled and analysed using a one-way ANOVA. The analysis showed significant differences ($F_{(8, 81)} = 99.03$, $\rho < 0.001$) between the nematode species, with regards to the mortality of the fourth- instar larvae of *B. impatiens* (Fig. 3.1). No significant difference in mortality was found for the fourth-instar larvae of *B. impatiens* with *S. yirgalemense* (87.00 % ± 3.96 %), *H. noenieputensis* (81.00 % ± 4.33 %), *H. indica* (84.00 % ± 4.52 %), *H. zealandica* (83.00 % ± 4.96 %), and *S. feltiae* (72.00 % ± 5.93 %), ($\rho > 0.05$), with the mean mortality ranging between 72 % and 85 %. *Heterorhabditis bacteriophora* had a lower mortality of 52.00 % ± 4.67 % in comparison to the five previously mentioned EPN species, but its lethal effect was not significantly different from that of *S. feltiae* ($\rho = 0.24$). The mortality was not significantly different between *Steinernema* sp., *S. jeffreyense,* and *S. khoisanae.* All three of these EPN species exhibited no insecticidal activity on the fourth-instar larvae of *B. impatiens*.

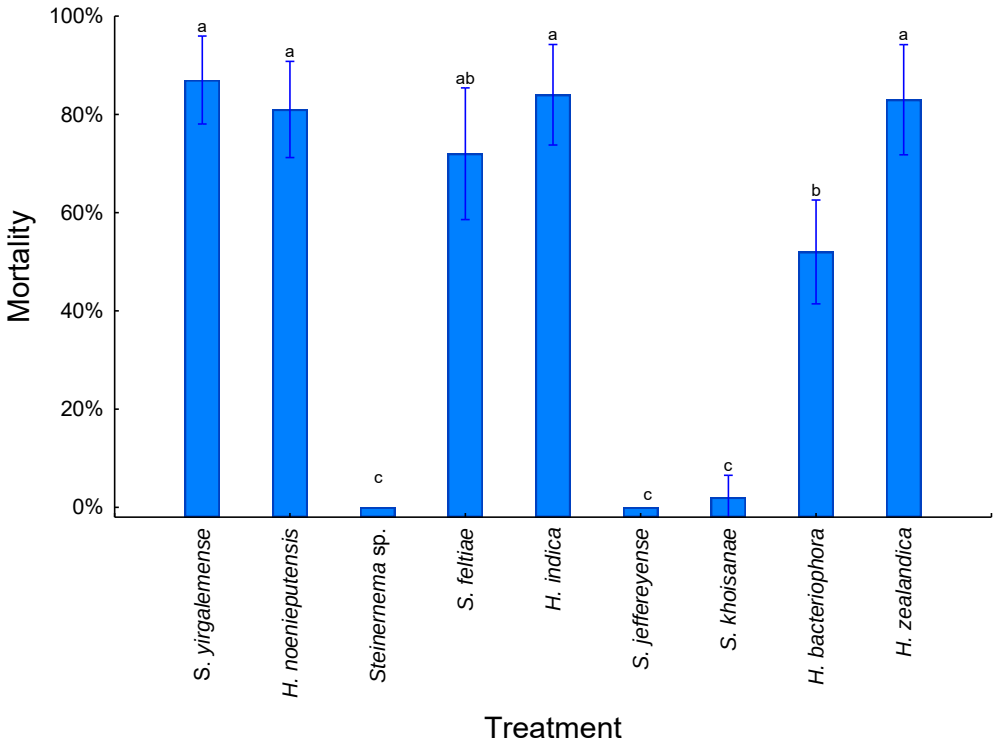

Figure 3.1. Mean percentage mortality (95 % confidence level) for fourth-instar *Bradysia impatiens* larvae after exposure to EPN species at a concentration of 100 IJs/larva (one-way ANOVA: $F_{(8, 81)} = 99.03$, $\rho < 0.001$). Different letters above the bars indicate significant differences between treatments.

Temperature trials

The results from the two-way ANOVA (with treatment and temperature as factors) performed to evaluate the mortality capacity of *S. yirgalemense*, *H. noenieputensis*, *H. indica*, *H. zealandica*, and *S. feltiae*, at different temperatures 13 °C, 25 °C, and 30 °C, are depicted in Fig. 3.2. Significant differences were obtained between the species at the different temperature levels ($\rho < 0.001$). The *S. feltiae* performed best at 13 °C (82.00 % ± 5.93 %), while the other species caused no mortality of the fourth instars at this temperature. At 25 °C, *S. yirgalemense* (87.00 % ± 3.96 %), *H. noenieputensis* (81.00 % ± 4.33 %), *H. indica* (84.00 % ± 4.52 %), and *H. zealandica* (83.00 % ± 4.96 %) had the highest mortality, while *S. feltiae* recorded a slightly lower mean percentage mortality (72.00 % ± 5.93 %), which was not, however, significantly different from that of *H. noenieputensis*, *H. indica*, and *H. zealandica.* At 30 °C, *H. indica* (85.00 % ± 5.22 %) had the highest mean, followed by *H. noenieputensis*

(84.00 % ± 4.52 %), *and S. yirgalemense* (79.00 % ± 5.86 %), which were not significantly different from *H. indica* and from *H. zealandica*, in contrast. *Steinernema feltiae* caused the least mortality on *B. impatiens* at this temperature, with a mean mortality of 47.00 % ± 6.84 %, as indicated in Fig. 3.2.

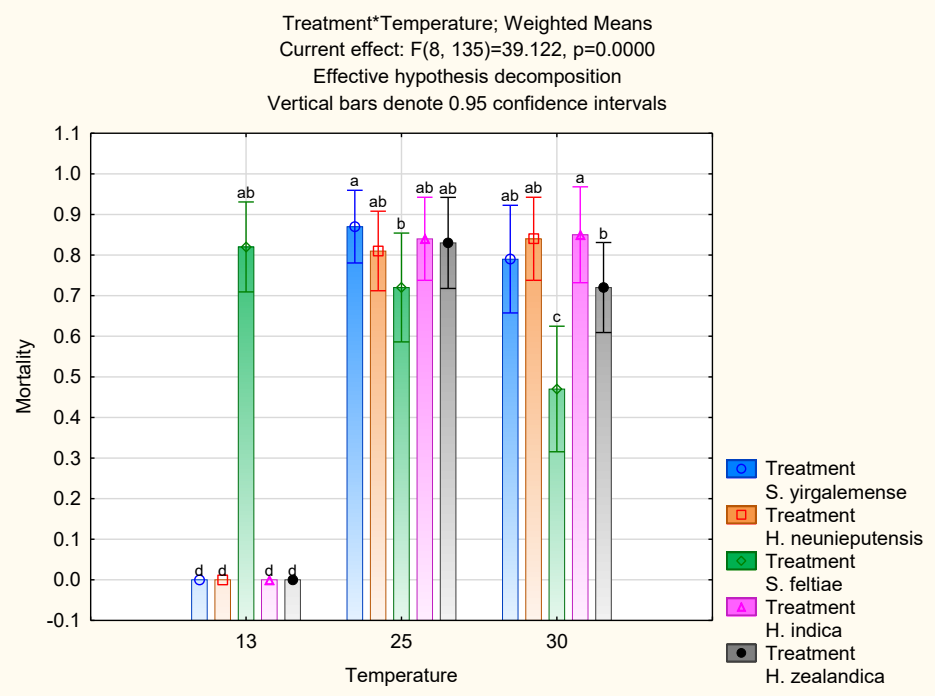

Figure 3.2. Mean percentage mortality (95 % confidence intervals) for fourth-instar *Bradysia impatiens* larvae after exposure to *S. yirgalemense* ( ), *H.noenieputensis* ( ), *S. feltiae* ( ), *H. indica* ( ), and *H. zealandica* ( ) at 13, 25, and 30 °C each, at a concentration of 100 IJs/insect (one-way ANOVA: $F_{(8, 135)} = 39.12$, $\rho < 0.001$). Different letters above the bars indicate significant differences between treatments.

LD_{50} and LD_{90} tests

A positive relationship was found to occur between the dosage and lethal capacity of the EPN species ($\rho = 0.014$), as shown in Fig. 3.3. At 5 IJs per larva, the mortality results for all the EPN species were significantly different from those for the control (*S. yirgalemense*, 36.00 % ± 4.61 %, *H. indica*, 46.00 % ± 4.61 %, and *H. zealandica*, 72.00 % ± 4.61 %). At the same dosage (5 IJs/larva), the percentage mortality caused by *H. zealandica* was very

high 72.00 % ± 4.61 %, and significantly different from the percentage mortality caused by any of the EPN species. Considering their mean percentage mortality, all three species achieved their highest insecticidal activity at 160 IJs/larva (*S. yirgalemense*, 96.00 % ± 4.615 %, *H. indica*, 100.00 % ± 4.61 %, and *H. zealandica*, 100.00 % ± 4.61 %). However, the mortalities concerned were not significantly different from those that were attained at the dosages of 40 and 80 IJs/larva (Fig. 3.3).

Results of the probit analysis showed increased mortality with increasing concentration of nematodes, with, however, the trends being slightly different for each of the EPN species ($\rho < 0.001$), (Fig. 3.3), suggesting that the lethal capacity for the different EPN species was different.

Results of the probit analysis showed the probit regression lines for *S. yirgalemense*, *H. indica* and *H. zealandica* to be different ($X^2 = 40.38$; d.f. = 4; $\rho < 0.001$). However, the probit regression line for *S. yirgalemense* was no different than was that of *H. indica*, having the same probit regression line as y = 1.50x + 3.57. The probit regression line for *H. zealandica* was y = 1.50 x + 4.37, with *H. zealandica* being found to be three times more potent than *S. yirgalemense*, *H. indica* (Fig. 3.3; Table 3.2). The data fitted this model very well ($X^2 = 46.09$; d.f. = 16), showing a positive relationship between nematode concentration and percentage mortality. *Heterorhabditis zealandica* had the lowest LD_{50} of 2.6 IJs per larva (90 % fiducial limits: 1.76-3.66) and an LD_{90} of 18.68 IJs/larva (90 % fiducial limits: 14.00-25.34), while *S. yirgalemense* and *H. indica* both had an LD_{50} of 8.98 IJs per larva (90% fiducial limits: 6.89-11.37 and 6.82-11.46 for *S. yirgalemense* and *H. indica*, respectively). The LD_{90} for *S. yirgalemense* and *H. indica*, respectively, were 64.16 (90 % fiducial limits: 49.41-87.53) and 64.13 (90 % fiducial limits: 48.99-88.22).

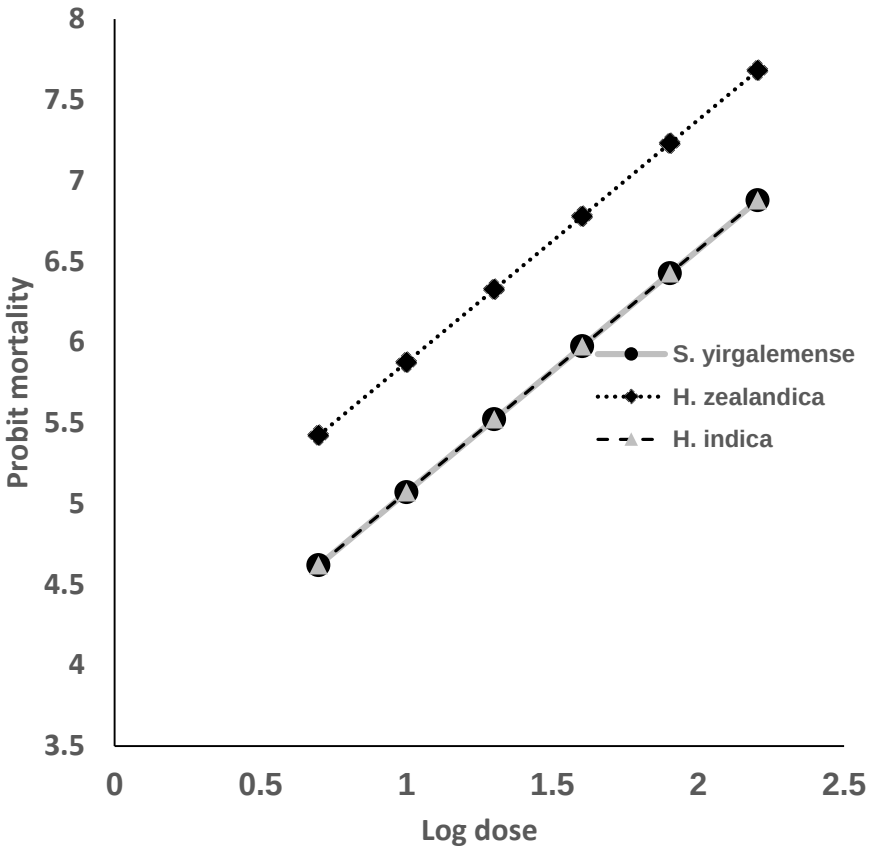

Figure 3.3 Probit mortality of fourth-instar larvae of *B. impatiens* at different concentrations (log dose) of *S. yirgalemense*, *H. indica*, and *H. zealandica;* 0, 5, 10, 20, 40, 80, and 160 IJs/larva (probit analysis).

Table 3.2. Relative potencies of different entomopathogenic nematode concentrations of *Steinernema yirgalemense, Heterorhabditis zealandica* and *H. indica* to control fourth-instar larvae of *Bradysia impatiens*.

Species	Relative to	Potency	95 % Fiducial limits
H. zealandica	*H. indica*	3.434	2.215 – 5.579
H. zealandica	*S. yirgalemense*	3.434	2.215 – 5.579
H. indica	*S. yirgalemense*	1.00	0.677 – 1.479

Temporal development of the EPNs in B. impatiens

The average number of IJs that penetrated each *B. impatiens* larva was 22 for *S. yirgalemense,* 19 for *H. indica*, and 17 for *H. zealandica*. All three EPN species took approximately one week to complete a successful life cycle inside the fourth-instar larvae of *B. impatiens* (Table 3.3; Fig 3.4). The number of EPNs and their growth stage were determined, as well as the sex ratio for *S. yirgalemense,* whose average was 1 male to 3 female adults. It was observed that, on the third day post infection, the adult EPNs depleted the food sources inside the larva, and the cuticle was seen to collapse. Upon emergence of the IJs, the petri dishes were transferred to White traps, and the average number of emerging IJs per larva was recorded. The average number of IJs to emerge per larva was 59 for *S. yirgalemense,* 22 for *H. indica*, and 41 for *H. zealandica* (Table 3.4).

Figure 3.4. Larvae of *B. impatiens* infected with IJs of *H. noenieputensis* (left) and IJs of *S. yirgalemense* emerging from infected larvae of *B. impatiens* (right).

Table 3.3. Temporal development of *Steinernema yirgalemense*, *Heterorhabditis indica* and *Heterorhabditis zealandica* in fourth-instar larvae of *Bradysia impatiens*, after inoculation with 100 IJs per larva.

EPN species	Days	EPN growth stage
S. yirgalemense	1	Immature/recovered IJs
	2	Adult males and females with eggs
	3	Adult males and females with eggs
	4	Adult males and females with larvae
	5	Adult males and females with larvae
	6	Infective juveniles
	7	Infective juveniles
H. indica	1	Immature/recovered IJs
	2	Immature/recovered IJs
	3	Adult hermaphrodites with eggs
	4	Adult hermaphrodites with larvae

	5	Adult hermaphrodites with larvae
	6	Infective juveniles
H. zealandica	1	Immature/recovered IJs
	2	Immature/recovered IJs
	3	Adult hermaphrodites with eggs
	4	Adult hermaphrodites with larvae
	5	Adult hermaphrodites with larvae
	6	Infective juveniles
	7	Infective juveniles

Table 3.4. Average penetration rate and production of infective juveniles for *Steinernema yirgalemense, Heterorhabditis indica*, and *H. zealandica.*

Species	Larval penetration	Infective juvenile production
S. yirgalemense	21.63	58.89
H. indica	19.38	22.38
H. zealandica	17.19	40.57

Discussion

Some *Bradysia* spp. have recently been shown to be among the most important pests of undercover crops. Control of the pests has, so far, been through the use of chemical pesticides, biological control means, and other physical practices (Cloyd 2015). However, Chandler *et al*. (2010) noted that, even though there are chemical insecticides that are labelled for use against fungus gnats, no pesticides have yet been registered for the control

of fly pests on herbs. Biological control measures for *Bradysia* spp. are specifically emphasised, due to two main reasons. Apart from being pests of crops, such as vegetables and mushrooms that are very close to consumption, *Bradysia* spp. are also pests of such crops as houseplants, which are found in very close proximity with humans (Gouge & Hague 1995b). Other reasons for biological control include the risk of *Bradysia* spp. developing resistance to chemical pesticides (Bartlett & Keil 1997), as well as the increasing demand by consumers for healthier products. Biological control has mainly been achieved through the use of EPNs and insect pathogenic bacteria. Control using EPNs has been dominated by the use of *S. feltiae*, which has biological constraints, and which has not been isolated from certain regions of the world. On the African continent, it has only been isolated from Algeria (Tarasco *et al*. 2009).

The current study had the objective of testing local EPN species for their ability to kill the larvae of *B. impatiens.* The species were identified in South Africa, in association with major tree nursery beds (Hurley *et al*. 2007a,b), and recently, in association with cucumbers, tomatoes, Chrysanthemums mushrooms, blueberries and other greenhouse herbs in the Western Cape province. Fourth-instar fungus gnat larvae, which have also been determined to be the most susceptible to EPNs (Gouge & Hague 1995a; Harris *et al.* 1995; Kim *et al.* 2004), were used in all the experiments undertaken. Results of the laboratory bioassays showed four local EPN isolates to have more than 80 % mortality efficacy. This is the first study to be undertaken in relation to the biological control of *Bradysia* spp. using South African EPNs.

The local EPN isolates that showed > 80 % mortality on *B. impatiens* included *S. yirgalemense*, *H. indica*, *H. zealandica, H. noenieputensis*, and *S. feltiae* at 25 °C. The results obtained did not differ in mortality from previous studies using *S. feltiae*. The latter EPN has been the most used species with regards to the control of *Bradysia* spp. The results obtained from this experiment shows the high potential for incorporating local EPNs into the biocontrol practices of *Bradysia* spp. in South Africa. However, simultaneously, it

poses a challenge for the undertaking of more biological studies with regards to the field performances of the local EPN species, and the optimisation of their mass culturing. Currently in South Africa, a total of 17 *Steinernema* and seven *Heterorhabditis* has already been reported (Malan & Hatting 2015; Malan & Ferreira 2017). Some of the EPNs concerned have already been tested for their potential to control key pests, such as codling moth, mealybugs, fruit flies, and the sugar cane stalk borer (De Waal *et al*. 2010; Malan *et al*. 2011; Ferreira & Malan 2014; Malan & Hatting 2015; Odendaal *et al.* 2016a,b), which have indicated promising results in this regard.

So far, special emphasis has been laid on *H. zealandica* and *S. yirgalemense*. The reason for this is that the two have shown potential in terms of their virulence against insect pests, as well as in terms of the ease of commercial mass production (Ferreira *et al*. 2015, 2016), as compared to the production rate that can be achieved with the other identified EPN species.

The results obtained with *S. feltiae*, as regards its insecticidal capacity on *Bradysia* spp., were no different from those that were obtained by means of past research that has been undertaken in terms of mortality. *Steinernema feltiae* has been determined to have a high lethal effect on *Bradysia* spp. (Harris *et al.* 1995; Gouge & Hague 1995b; Mansilla & Pastoriza 2001). During our temperature trials, *S. feltiae* registered a relatively low lethal capacity, at 30 °C. The trial results in question did not differ from those that have been presented by other researchers who have cited biological limitations for *S. feltiae* at higher temperatures (Gouge & Hague 1995a,b; Jagdale *et al*. 2004). For example, Gouge & Hague (1995a) tested different steinernematid and heterorhabditid species to determine their infectivity and insecticidal capacity on sciarid flies. In this study, *S. feltiae*, though being the most effective for controlling sciarid species, was less effective at higher temperatures. The UK isolates of *S. feltiae* were also shown to be more effective against UK sciarids than were the other EPN isolates tested from other European countries (Gouge & Hague 1995a). In another experiment, Jagdale *et al.* (2004) indicate that *S. feltiae* gave better results in a

growth chamber (22 °C), achieving a higher control efficiency of 73 % to 80 %, as compared to only 34 % to 41 % in the greenhouse, where the temperatures were generally higher (above 25 °C).

The LD_{50} and LD_{90} for *H. zealandica* (2 and 23) was less compared to those for *S. yirgalemense* (8,65) and *H. indica* (8,59) indicating that *H. zealandica* required fewer IJs to penetrate the insect larva so as to be able to kill larvae of *B. impatiens*. Different *H. indica* strains have been reported to show a high percentage of lethal capacity to fungus gnat larvae, in terms of both laboratory and field trials (Leite *et al*. 2007).

Heterorhabditis zealandica has recently been used in other laboratory bioassays, showing greater potential as a biocontrol agent than do other South African EPNs. For example, Le Vieux and Malan (2013) and Stokwe and Malan (2015) compared *H. zealandica* and other local EPNs for their potential to infect mealybugs *Planococcus ficus* Signoret and *Pseudococcus viburni*, Signoret respectively. In both the experiments concerned, *H. zealandica* emerged as the better alternative. This is an advantage for both *S. yirgalemense* and *H. zealandica,* since they have been shown to be relatively easy to mass culture (Ferreira *et al*. 2015, 2016). The average number of IJs that entered the larvae was higher for *S. yirgalemense* (22) than it was for either *H. zealandica* (17) or *H. indica* (19). The finding conflicts with the results that were obtained by Le Vieux & Malan (2013) and Stokwe and Malan (2015), who found *H. zealandica* to have a greater penetration potential than did *S. yirgalemense* for mealybugs.

As regards the temporal development of the EPN in the fourth instar of *B. impatiens*, the results showed that *H. zealandica, S. yirgalemense* and *H. indica* were able to complete their life cycle within the period of one week. Adult EPNs were observed inside the cadavers on the second day, while first-generation J1s were observed on the fourth day, for all the species concerned. Such rapid development of EPNs within the sciarid larvae did not differ from the observations made by Gouge & Hague (1995c), who reported that *S. feltiae*

developed faster in relation to sciarid larvae than did the other hosts tested. Gouge and Hague (1995c) also note that only one generation of the *S. feltiae* was present in the *B. paupera* larvae, as was also found in the present study.

The size of IJs in relation to the size of the insect concerned has been indicated to be a determining factor in terms of the EPN´s ability to infect a certain insect by Bastidas *et al*. (2014). Their study concluded that the ability of the EPNs to invade insect micro hosts decreased with increasing EPN length and decreasing host size. The results of the current study agree with this assertion. The relatively bigger nematodes, namely *S. jeffreyense*, *S. khoisanae*, and *Steinernema* sp., were unable to infect the fourth-instar larvae of *B. impatiens*, whereas the smaller nematodes, *S. yirgalemense*, *S. feltiae*, *H. bacteriophora*, *H. noenieputensis*, *H. zealandica*, and *H. indica*, were all able to infect the larvae of *B. impatiens*, causing significant mortality. As has already been indicated, IJs tend to enter the sciarid larvae through the mouth and anus (Gouge & Hague 1995b,c). Their use of such an entrance pathway is attributed to the small size of the larva spiracles that limits the entry of the nematodes (Gouge & Hague 1995c). Renn (1998) indicated that the smaller openings on the larvae tended to impede nematode invasion.

The results of the current experiment show that, under optimum conditions, the locally isolated EPNs, namely *H. zealandica, S. yirgalemense*, and *H. indica*, tend to have great potential for use as biocontrol agents for *B. impatiens*. By means of the use of bioassay protocols, a percentage mortality of > 80 % was achieved for the above-mentioned species at 25 °C and 30 °C, which supports their ability to be used in local greenhouses, where the average temperature normally exceeds 25 °C. However, additional research should be undertaken into the effect of timing of EPN applications, and into effective EPN concentrations and application techniques, among other factors, to provide further critical information regarding the field applications of such nematodes.

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Chapter 4

Greenhouse application of *Steinernema yirgalemense* to control fungus gnats, *Bradysia impatiens* (Diptera: Scaridae)

Abstract

Bradysia impatiens Johannsen has recently been identified as a pest, in association with major tree nursery beds, cucumber, mushrooms, blueberries and tomato greenhouses, Chrysanthemums and other greenhouse-cultured herbs in South Africa. This study had the objective of determining the effect of different concentrations of a local entomopathogenic nematode, *Steinernema yirgalemense,* for its potential to control a natural infestation of *B. impatiens* in a commercial cucumber greenhouse. Additionally, the effect of potting media on nematode movement to control an artificial population of fungus gnats was tested. The concentrations used were based on the recommended concentration of *S. feltiae* (a commercial product) for the control of sciarids (5×10^5 IJs per m^2). Doubling the number of nematodes in the recommended dosage reduced the number of fungus gnats by 77 % after 14 days, and by 76 % after 21 days. However, the fungus gnat populations were observed to restore quickly to their original levels, after a period of approximately three weeks, emphasising the short life cycle of the fly, as well as the importance of the pre-treatment, and of consecutive applications, of EPNs. The three different types of potting media, consisting of pine sawdust, coco coir, and a mixture of both bale coir and vermiculite, all had a positive effect on the movement of *S. yirgalemense.* A mortality of > 75 % was achieved for the fungus gnat larvae that were added to the media, as indicated by the number of adult fungus gnats caught on the yellow sticky cards used. The results show both the successful and the positive prospects for using the local *S. yirgalemense* under glasshouse conditions.

Introduction

Bradysia spp. are some of the major pests that are capable of causing significant economic losses in crops in protected environments (Jagdale *et al.* 2004; Chandler *et al.* 2010; Vaughan *et al.* 2011). *Bradysia* spp., which are also commonly known as fungus gnats, or mushroom flies, or nuisance flies, are frequent pests in greenhouses, nursery beds, houseplants, and mushroom compost (Harris *et al.* 1995; Mansilla & Pastoriza 2001; Vänninen 2003; Kim *et al.* 2004; Mohrig *et al.* 2013; Villanueva-Sánchez *et al.* 2013). They have been referred to as generalist opportunistic herbivores that usually feed on organic matter and fungi, but which resort to eating roots and underground stem tissue in the absence of their normal feed (Vaughan *et al.* 2011; Shin *et al.* 2013). However, a recent study by Lee *et al.* (2010) mentions that the trend concerned could have been altered, as in the case of what the study classified as living plant-feeding larvae, with “the hypothesized larval habitat of Sciaridae shift[ing] during the course of evolution from an ancestral type of mixed dead plants to living plants.” Accordingly, larvae can survive on living plant parts that have been attacked by fungi.

The effects of fungus gnats include the direct physical damage that they wreak on plants by means of their feeding and tunnelling into plant tissue, including the roots, root hairs, stems, and leaves (Springer 1995; Mansilla & Pastoriza 2001; Vänninen 2003), and the indirect damage that they cause through transferring fungal diseases and creating entry points for plant pathogens (Pundt 1999; Ludwig & Oetting 2001; Scarlett *et al.* 2014). Adult fungus gnats, though physically harmless, transmit plant pathogens (Pundt 1999) and swarm farm workers, causing them discomfort (Schuhli *et al.* 2014). Larval feeding on roots can result in significant damage to the root system and notable root biomass loss (Springer 1995; Cloyd & Zaborski 2004). The symptoms presented by affected plants include wilting, loss of vigour, reduced vegetative development, abraded roots, and the loss of leaves, or even plant death, where heavy fungus gnat infestations occur (Springer 1995; Pundt 1999; Mansilla & Pastoriza 2001; Cloyd & Zaborski 2004). The effects of pests, reduce plant yield and largely

increase the amount of production costs involved in terms of pest control (Popp & Hantos 2011).

The biological control of fungus gnats, using entomopathogenic nematodes (EPNs), has resulted in economic gains, especially in terms of plant plug production, where sciarid infestation would, otherwise, be likely to prevent the plugs from developing roots. EPNs belong to the order Rhabditida, with Steinernematidae and Heterorhabditidae being the two most commercialised families for use against insect pests. Such commercialisation is the outcome of their remarkable success in managing insect pests, as compared to other families. In particular, the two families concerned, which have emerged as excellent biocontrol agents of soil-dwelling insect pests, are now used to control a wide range of foliar, soil surface, cryptic, and subterranean-dwelling insect pests (Lacey & Georgis 2012). The use of EPNs has been expedited through the discovery of multiple efficacious strains and the desire to reduce pesticide usage (Lacey & Georgis 2012). EPNs are currently being used in the biological control of certain insect pests, mainly because of their ease of mass production, their broad host range, and the degree of safety for both mammals and the environment with which they can be used (Kerry & Hominick 2002). Their dominance is also credited to the advances that have been made in their commercial production and formulation technology. The successful use of EPNs, however, is influenced by a large number of complex interactions between animals, plants and the environment (Kerry & Hominick 2002).

EPNs can be applied in conjunction with certain chemical pesticides, soil amendments and fertilisers. The infective juveniles (IJs) have been found to tolerate short exposures (2 h to 6 h) of most acaricides, fungicides, herbicides, and insecticides (Rovesti & Deseö 1990; Rovesti *et al*. 2013; Laznik & Trdan 2014). Such tolerance levels supports their ability to be used in integrated pest management practices. However, certain studies have shown that some pesticides can reduce the prevailing levels of EPN viability and infectivity (Zimmerman & Cranshaw 1990; Alzugaray 1991; Krishnayya & Grewal 2002; Hazir *et al.* 2004; Rovesti *et*

al. 2013; Echegaray *et al*. 2015). The above implies that a deliberate effort should be made to determine the compatibility of the EPNs and the chemicals concerned, at the time of application.

The IJ is the only free-living stage of EPNs that is capable of movement outside of the host in the soil, in search of a new host to infect. The IJ enters through the insect's natural openings, including the mouth, anus, and spiracles. The IJs of *Heterorhabditis* species can penetrate through the cuticle and then directly enter the insect's haemocoel, because they possess an anterior tooth that is adapted for the purpose (Kaya *et al.* 1993; Nobuyoshi 2002). The nematodes feed, develop and reproduce within the insect cadaver. The IJs feed on their symbiotic bacterial cells that they release into the haemocoel and decayed host tissues (Nobuyoshi 2002). Under ideal conditions, the IJs begin to exit from the cadaver from 7 to 15 days after infection, whereupon they begin to search for new hosts (Kaya *et al.* 1993).

Most of the studies that have been done regarding the control of *Bradysia* spp. using EPNs point to the use of *Steinernema feltiae* Filipjev, Wouts, Mráček, Gerdin & Bedding 1934 (Harris *et al*. 1995; Gouge & Hague, 1995a,b; Vänninen 2003, Jagdale *et al*. 2004; Jess & Bingham, 2004). *Steinernema feltiae* has been chosen for its high efficiency to control sciarids, with satisfactory results having been achieved in this regard. However, the use of only *S. feltiae* as the ultimate EPN for fungus gnat control poses challenges for use in certain countries, especially where the species has not previously been isolated. *Steinernema feltiae* has also been shown to have biological limitations, especially at relatively high temperatures (Gouge & Hague, 1995a,b; Jagdale *et al.* 2004). Although having a worldwide distribution, on the African continent, *S. feltiae* has only been isolated from Algeria (Tarasco *et al*. 2009). Such a distribution pattern has created the need to use locally isolated EPN species that are better adapted to the local environment than are other species. This is even more the case because the introduction of foreign strains into a country should be properly controlled, since otherwise it might adversely affect the existing environment (Alpert 2006).

As regards the field application of EPNs, certain factors, such as crop and substrate type, nematode concentration, the timing and method of application, and soil texture have been determined to affect the efficacy of EPNs. Potting medium has been discussed as affecting nematode movement, as well as fungus gnat larvae movement and colonisation (Jagdale *et al.* 2004), while different EPN concentrations have been used for controlling fungus gnats, depending principally on the prevalent crop cover and fungus gnat populations.

Bradysia impatiens Johannsen has been identified in South Africa, in association with major tree nursery beds (Hurley *et al*. 2007a,b), and, recently, in association with cucumbers, tomatoes, Chrysanthemums, mushrooms, blueberries and other greenhouse herbs in the Western Cape province (Katumanyane *et al*. 2017a,b). The objective of the current study was to determine the effect of different concentrations of locally isolated *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, for its potential to control *B. impatiens* in a commercial cucumber greenhouse. Additionally, the effect of potting media on nematode movement for the control of fungus gnats was tested. *Steinernema yirgalemense* was chosen for the field trials, because of its comparably high virulence, obtained during the screening against *B. impatiens*. *Steinernema yirgalemense* is currently the species of choice, with it having already been successfully mass cultured *in vitro* in liquid culture, and formulated on a small scale, which is currently being upscaled to fermenter size.

Materials and methods

Source of insects

A culture of *B. impatiens* was raised and maintained in the laboratory at room temperature, (25 °C) using a 3:1:1 ratio of partially decomposed pinewood chips, soymeal and cornmeal, respectively. Late third instar larvae of *B. impatiens* were used in the experiments. The larvae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) were initially obtained from a pet shop, and afterwards cultured in vented plastic containers on fine bran and carrots.

Source of EPNs

Steinernema yirgalemense was obtained from the EPN collection of the nematology laboratory at the Department of Entomology and Conservation Ecology of Stellenbosch University, where it is currently being stored as isolate number 157-C. Freshly harvested IJs were obtained through culturing in mealworms, *T. molitor* L. in a growth chamber at 25 °C. Modified White´s traps (White 1927) were used to harvest the EPNs, for a period of one week after emergence. Harvested IJs were then stored in distilled water at 12 °C, in horizontally placed culture flasks (Woodring & Kaya 1988). The stored EPNs were shaken periodically for ventilation and used within three weeks after EPN harvest to ensure nematode viability.

Effect of potting media

Three organic potting media, pine sawdust (local sawmill, untreated), coco coir (Greenhouse Technologies), and a mixture of coir bale (Greenhouse Technologies) and vermiculite (Hygrotech sustainable solutions), were used. The potting media were obtained from greenhouse farmers located in the Western Cape province. The experiment was carried out in a commercial cucumber greenhouse in the Western Cape. Similar volumetric measures of each substrate were used. The ambient temperature and the relative humidity (RH), as well as the temperature of the substrate, were recorded daily. For each substrate, 10-L plastic grower bags (20) were filled with moist substrate. Two-week-old, mini-cucumber plantlets (var. Kaspin), grafted on pumpkin stalks (var. Ferro), were planted in each bag and left to establish for one week. Ten pots were used as treatment for each of the substrates, while 10 pots of each substrate were used as the control. The pots were arranged in a completely randomised design. Each of the experimental pots was isolated, using white organza fabric that was tightly wrapped around the pot and tied at 1.5 m atop the plant with a plastic string, while it was tightened at the bottom of the grower bag using packing tape, as is shown in Figure 4.1.A. The organza fabric sides were sealed, using double-sided tape. Such

treatment, which made the setup insect-proof, prevented the adult fungus gnats from escaping, while leaving them enough space to move around freely.

Yellow cards (15 × 10 cm), covered with glue on both surfaces, were supported with bamboo skewer sticks. A card was placed in each of the pots, at about 5 cm from the surface of the substrate, to attract and trap any emerging adult fungus gnats, as is shown in Figure 4.1.B. Ten pots of each substrate treatment were sprayed with the IJs of *S. yirgalemense*, at a rate of 5×10^5 IJs / m^2 in 500 ml of water, while the control received water only. This allowed the IJs to move freely in search of possible hosts, and to establish within the substrate. Twenty-four hours after inoculation with nematodes, all the pots (n = 60) were inoculated with approximately 70, late third instar larvae of *B. impatiens*. Before adding the larvae of *B. impatiens*, the substrates were moistened. A small opening was made on the substrate surface close to the root zone, into which the insect larvae were inserted, and which was then covered over with a small layer of the substrate. The pots were watered constantly to keep them at 100 % moisture, with both watering and fertilisation being done from underneath the grower bag, so as to minimise any disturbance of the setup. The plants were then left undisturbed for 10 days, after which the number of adult fungus gnats on each sticky card was recorded.

Figure 4.1. A) Insect-proofing set up to contain emerging *Bradysia impatiens* adults. B) Yellow sticky card, supported on bamboo skewer sticks, so as to trap emerging fungus gnats.

Effect of nematode concentration

The efficacy of three EPN concentrations of *S. yirgalemense* of 2.5×10^5, 5×10^5, and 10×10^5 IJs per m^2 were tested for their ability to control *B. impatiens* in a commercial cucumber greenhouse, with a natural high infestation of fungus gnats. The concentrations were based on the recommended concentrations of EPNs of the product nemaplus® (*S. feltiae*) for the control of sciarids. Each of the cucumbers (var. Litoral) was grafted onto a pumpkin rootstalk (var. Ferro). Ten pots were used for each treatment concentration, with 10 pots being used for the control. The corresponding concentration of EPNs was drenched onto the surface of the pot in 500 ml of water, while the control received 500 ml of water only. The pots were immediately irrigated to wash the nematodes into the substrate. The trial pots were each isolated by means of a covering of white organza fabric, up to a height of 1 m, as described above. However, in this particular setup, the piece of white organza fabric used in each case was tied below the leaf area of the cucumber plants, since this was an established plantation, with relatively tall plants, as is shown in Figure 4.2. The experimental

setup described prevented the adult fungus gnats from escaping, but left enough space for their free movement. After one week, a 15 × 10 cm yellow sticky card, with glue on both surfaces, and fitted on bamboo skewer sticks, was placed in each pot, at a height of about 5 cm from the surface of the medium, so as to attract and trap any emerging adult fungus gnats. The sticky cards were added after one week to allow the already existing adult fungus gnats and the pupae to emerge and die before the EPNs could have an effect. The experiment was then monitored weekly for four weeks, during which time the number of adult fungus gnats that had become stuck on each sticky card was recorded. The sticky cards were replaced weekly, with new sticky cards being provided after each reading. The experiment was repeated using a fresh batch of nematodes on a different test date. The soil and ambient temperature, as well as the ambient RH, were measured daily throughout the experiment.

Figure 4.2. Experimental setup made insect-proof by means of a piece of white organza fabric, tied with a string at a height of about 1 m, and tightened to the grower bag using packaging tape, with the sides sealed with double-sided masking tape.

Statistical analysis

Statistical analyses were conducted using Statistica 13.2 software (StatSoft Inc. 2016) to determine the effect of potting media on EPN movement, and the effect of *S. yirgalemense* concentration on fungus gnat mortality. The data were analysed, using ANOVA, to compare the mean mortality, with a post hoc comparison of means being applied using Bonferroni's method, in the case of significant differences existing between the test dates and treatments concerned. A bootstrap multi-comparison was performed (Efron & Tibshirani 1993), in case the residuals were not normally distributed. The test trials for the effect of EPN concentration on fungus gnat mortality were repeated by means of a fresh batch of nematodes on a different test date. If no significant differences were found between the main effects of treatment and the date, the data from the two repeats were pooled before analysis. The data obtained were expressed as weighted means ± standard error to establish the effect of the potting media, while least square means were used for the field application of the EPNs.

Results

Effect of potting media

During the 10 days of the experiment, the ambient temperature in the greenhouse oscillated about an average of 24.2 °C. The temperatures varied between a minimum of 9.6 °C, recorded in the small hours of the morning, to a maximum of 57.6 °C, recorded in the afternoon. The trend in the ambient RH was seen to act in reverse relationship to the temperatures, increasing while the temperatures reduced, and vice versa (Fig. 4.4.A). The RH oscillated between a minimum of 15.2 % recorded in the afternoon, and a maximum of 95.8 % recorded in the morning. The average RH recorded during the 10-day period was 66.1 %. However, the temperature of the medium only rose to a maximum of 30.6 °C in the afternoon, while maintaining an average of 23.0 °C (Fig. 4.4.B). The minimum temperature recorded for the medium was 16.6 °C during the early morning hours.

A one-way ANOVA to test for the effect of the EPN application on the different substrates (treatment) used to control *B. impatiens* showed significant differences between the treatments and the control ($\rho < 0.001$), with the former showing significantly fewer adult fungus gnats becoming stuck on the yellow sticky cards throughout the trial period. No significant differences were found between the substrate types ($F_{(2, 54)} = 1.7516$, $\rho = 0.183$) with regards to the fungus gnat mortality, implying that the substrates had a similar effect on the movement of *S. yirgalemense* in terms of the larva control of *B. impatiens*. The three substrates showed a mean mortality that was significantly higher than was that of their respective control treatments ($\rho < 0.001$), indicating that none of them affected the movement of *S. yirgalemense*. The mixture of coir bale and vermiculite substrate in the treatment experiment had the highest mortality mean (78.71 % ± 3.13 %), followed by that of the pine sawdust (77.14 % ± 2.85 %), and then, lastly, by that of the coco coir (75.57 % ± 3.07 %). In the control, the pine sawdust (22.57 % ± 7.70 %) had the least mortality mean for *B. impatiens* larvae, followed by that of the coir bale plus vermiculite mixture (35.29 % ± 4.16 %), which, however, was not significantly different from that of the coco coir (39.14 % ± 4.32 %), in contrast, as is shown in Figure 4.3.

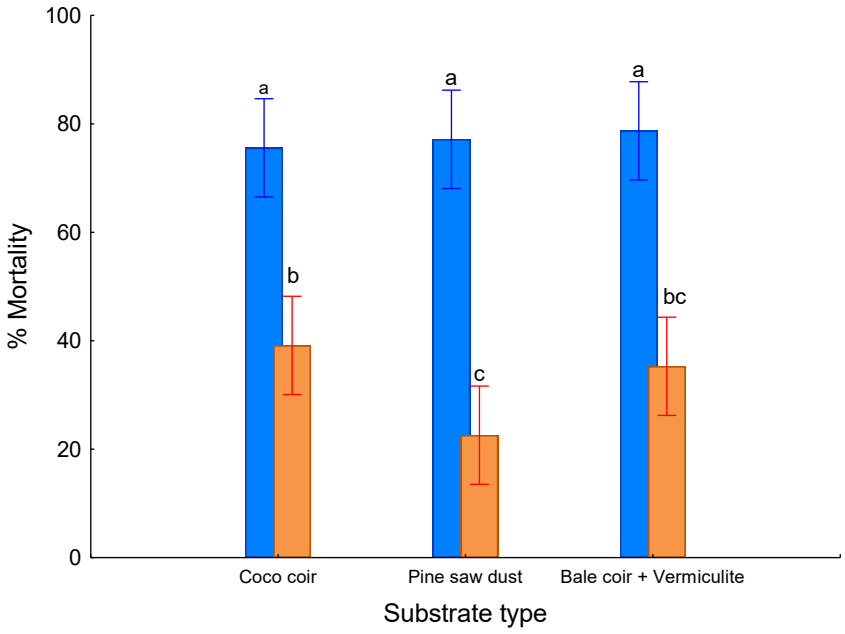
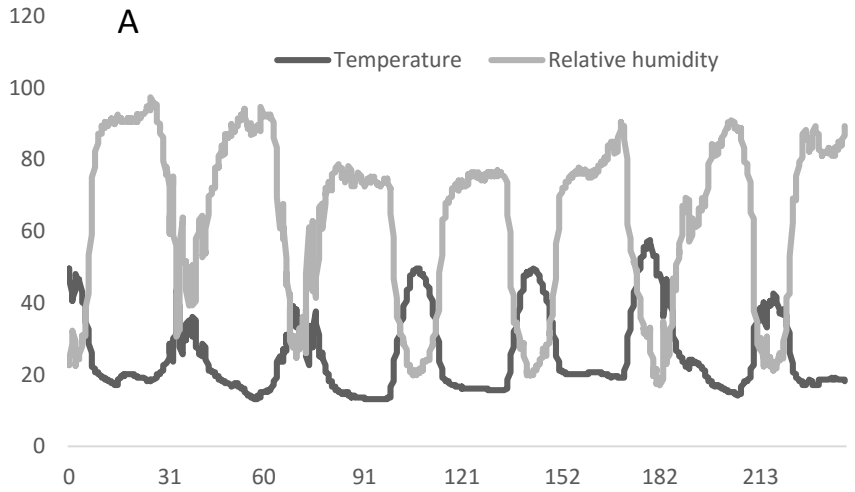


Figure 4.3. Mean percentage mortality (95 % confidence intervals) for fourth instar larvae of *Bradysia impatiens* larvae in different substrates after exposure to *Steinernema yirgalemense* at a concentration of 5 × 10^5 IJs/ m^2 (two-way ANOVA: $F_{(2, 54)} = 2.0482$, ρ = 0.13887), with treatment ( ), and control ( ). Different letters above the bars indicate significant differences between the treatments.



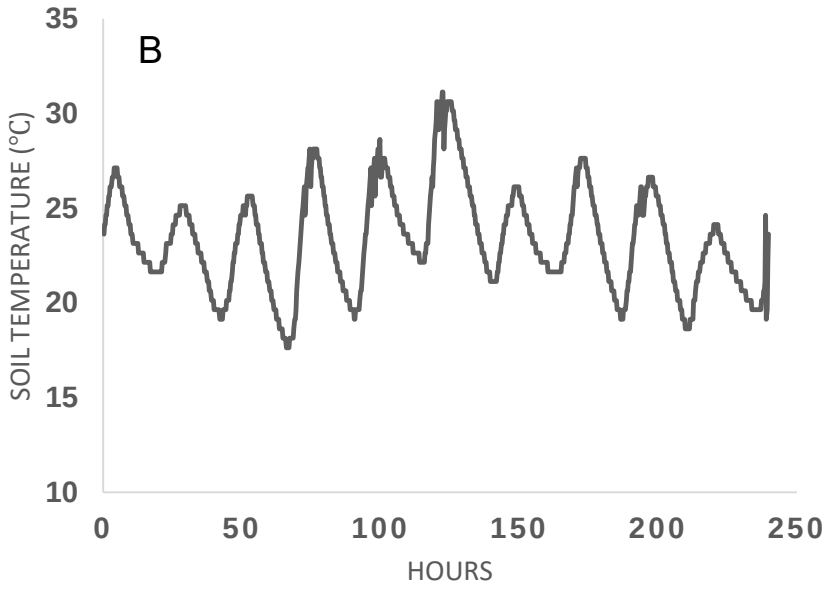


Figure 4. 4. A. Ambient temperature and relative humidity data recorded in the greenhouse during the 10-day period. B. Soil temperature data recorded during the 10-day period.

Effect of nematode concentration

During this experiment, the ambient temperature in the greenhouse fluctuated between 9.6 and 60.0 °C, with an average of 22.8 °C. The trend of the ambient RH was opposite to that of the temperature. The RH humidity decreased in contrast to a rise in temperature, and vice versa. The RH oscillated between a minimum of 13.5 %, recorded in the afternoon, and a maximum of 98.2 %, recorded in the early morning. The average RH recorded during the 28 days was 67.3 %. In the medium, the temperatures only reached a maximum of 30.1 °C in the afternoon, while maintaining an average of 21.0 °C overall. The minimum temperature recorded for the medium was 12.1 °C in the early morning hours.

The results of the two-way ANOVA testing for the effect of time and of the concentrations of *S. yirgalemense* on the mortality of fungus gnats over a period of 28 days showed no significant interaction between the two main effects ($\rho = 0.152$). A one-way

ANOVA analysis (Fig. 4.5) showed that the mortality of fungus gnats, as indicated by the numbers of adult flies found on the yellow sticky cards, increased significantly with increasing EPN concentrations ($F_{3, 228} = 24.775$, $\rho < 0.001$). After a period of 28 days, the control experiment (T0 = control experiment) was found to have the highest number of fungus gnats on the sticky cards involved (13.37 ± 0.78), which was statistically significant from all the other treatments (Fig. 4.5). The concentration of 10×10^5 IJs per m^2 (T3) showed the lowest mean of fungus gnats (4.05 ± 0.78), signifying a higher percentage control (approximately 70 %) as compared to the concentration of 2.5×10^5 IJs per m^2 (T1) (10.35 ± 0.78), and 5×10^5 IJs per m^2 T2 (8.42 ± 0.78), which did not differ significantly from each other (Fig. 4.5).

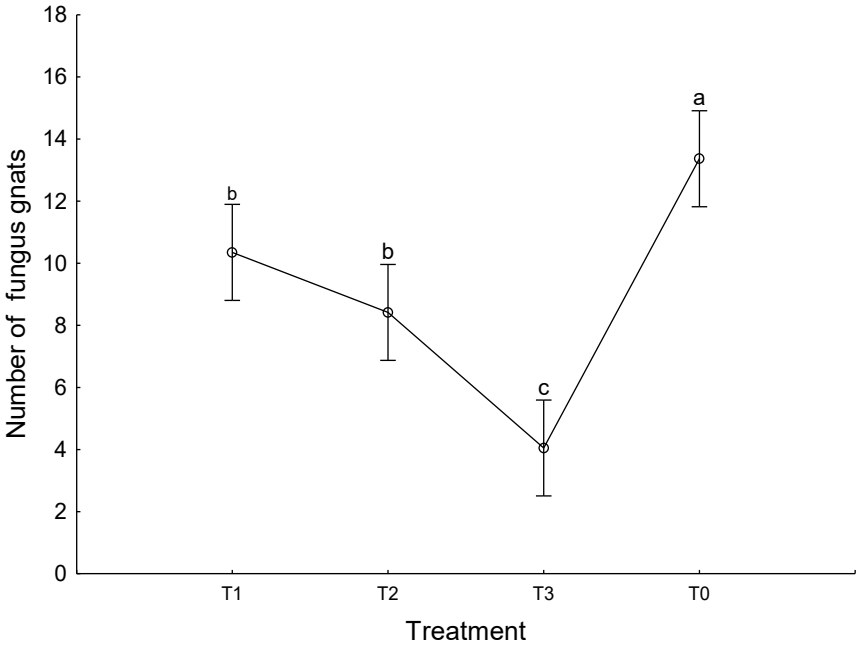


Figure 4.5. Mean number of adult *Bradysia impatiens* (fungus gnats) (95 % confidence intervals) that were recovered from the yellow sticky cards, 28 days after applying different concentrations of *Steinernema yirgalemense* (T1 = 2.5 × 10^5 IJs / m^2, T2 = 5 × 10^5 IJs / m^2, T3 = 10 × 10^5 IJs / m^2), while T0 is the control treated with water only (one-way ANOVA: $F_{(3, 228)} = 24.775$, $\rho < 0.001$). Different letters above the bars indicate significant differences between the treatments.

At a period of 14 days after spraying with nematodes, the concentration of 10 × 10^5 IJs / m^2 (T3) showed the lowest number of adult fungus gnats to be retained on the sticky cards (3.20 ± 1.36) (Fig. 4.6). However, over time, a general increase occurred in the mean number of fungus gnats on the sticky cards for all the treatments concerned. After 28 days, no significant differences were found between T1 (11.50 ± 1.36), T2 (11.00 ± 1.36) and the control (11.80 ± 1.36) (Fig. 4.6). Doubling the concentration of nematodes from the recommended number of 5 × 10^5 IJs / m^2 to 10 × 10^5 IJs / m^2 reduced the number of fungus gnats by 77 % after 14 days, and by 76 % after 21 days.

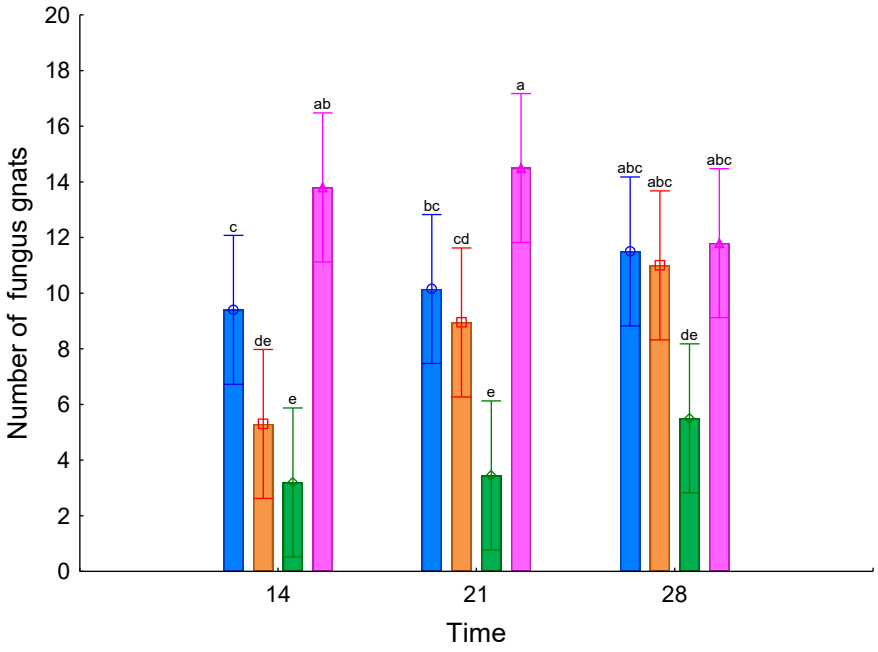


Figure 4.6. Mean number of adult *Bradysia impatiens* (fungus gnats) (95 % confidence intervals) that were recovered from the yellow sticky cards after a period of 14, 21 and 28 days, after applying different concentrations of *Steinernema yirgalemense* of T1, ( ) = 2.5 × 10^5 IJs / m^2, T2, ( ) = 5 × 10^5 IJs / m^2. T3, ( ) = 10 × 10^5 IJs / m^2, while T0 ( ) was the control experiment treated with water only (one-way ANOVA: $F_{(6, 228)}$ = 1.5862, ρ = 1.52). Different letters above the bars indicate significant differences between the treatments.

Discussion

The type of growth medium is one of the numerous abiotic factors that limit the survival and efficacy of EPNs. The suitability of the medium for EPN survival has been shown to depend mainly on the texture, the structure, the moisture, and the temperature, among other factors. A recent review by Griffin (2015) indicated that, even though many studies have been done with regards to the effect of soil properties on the efficacy of EPNs, scant information exists on the behaviour of EPNs in soilless, organic growing media. In the case of fungus gnats, management of the potting medium affects both nematode movement and fungus gnat larvae movement and colonisation (Jagdale *et al.* 2004). Different EPN species

have also shown different foraging behaviour for the different types of organic growth media (Nielsen & Lewis 2011).

The culture media can have an effect on fungus gnat proliferation and can, as well, be a source of introduction of fungus gnats into the farm (Cloyd & Zaborski 2004). The selection of growing media can, thus, be used as a management tool, as the fungus gnat female adults tend to be more attracted to certain media than they are to others, for purposes of egg-laying (Cloyd *et al*. 2007). Continuously decomposing organic media have been shown to increase their water- holding capacity and to reduce their porosity, which increases the fungus gnat population and suppresses the behaviour of several other soil organisms, including EPNs. Unfortunately, the currently advocated substrates, such as bark, wood fibre, coir, and composted green waste, seem to be more associated with fungus gnats than is the more conventional peat (Chandler *et al.* 2010). As manipulation of the soil environment can enhance EPN efficacy, growers considering incorporating EPNs in their pest management programme should endeavour to use the media that are best suit the purpose.

In the current experiment, the three different types of potting media that was tested, consisting of pine sawdust, coco coir and a mixture of both coir bale and vermiculite, all had a positive effect on the movement of *S. yirgalemense.* Higher than 75 % mortality was achieved for the fungus gnat larvae that were added to the media, as was indicated by the number of adult fungus gnats that were caught on the yellow sticky cards. However, the number of insects that were trapped by the sticky traps might not be an exact indication of the number of fungus gnats present (Harris *et al.* 1995; Vänninen 2003). The control for the pine sawdust showed a significantly higher number of adult fungus gnats on the sticky cards in comparison to those used in the controls of the other media. Such a high rate could be attributed to the fact that the substrate in question was obtained from the farm with a heavy sciarid infestation, which could, ultimately, have been a secondary source of fungus gnat infestation.

Conducting both adult sciarid and larvae monitoring is important to enabling the approximation of fungus gnat populations, and to ensuring the timely control of fungus gnats, since, as has been discussed, in cases of heavy infestation, high numbers of sciarids have tended to overwhelm the nematode numbers applied. However, currently, no threshold numbers exist for the application of management strategies for fungus gnats. As regards the application of EPNs, different concentrations may be required to achieve a similar percentage of fungus gnat control for different crops (Jagdale *et al.* 2004). This is because different crops, as well as different vegetative cover, have different susceptibilities to fungus gnat populations. Plants with relatively high amounts of ground vegetative cover are likely to intercept more EPNs from reaching the medium than might otherwise be the case. In contrast, crops that are more susceptible to fungus gnat attack would tend to have higher fungus gnat populations that would require higher concentrations of EPNs for their control, since a high number of fungus gnats could easily overwhelm the nematode numbers concerned. Different researchers have used various concentrations in field trials for the control of fungus gnats. For example, Jagdale *et al.* (2004) used 5×10^5 IJs / m^2 of *S. feltiae*, achieving 73 to 80 % control of *Bradysia coprophila* in growth chamber-grown poinsettia (between 17 and 45 days after treatment). However, they achieved a lower percentage control of 34 % to 41 % for *B. coprophila* on greenhouse-grown poinsettia (between 17 and 45 days after treatment), using the same concentration of nematodes. Jess & Bingham (2004) used 3×10^6 IJs / m^2, achieving > 80 % control of *Lycoriella ingenua* in mushroom compost and in casing substrates, while Harris *et al.* (1995) achieved about the same percentage control (higher than 80 %) using a much lower concentration of 1×10^5 IJs / m^2 on *Bradysia agrestis* Sasakawa in Jiffy plugs of poinsettia cuttings.

In the current study, doubling the recommended concentration of EPNs to control a high fungus gnat infestation on a commercial cucumber farm was able to give significant results during the one-month period of the experiment. The significant fungus gnat control achieved with such a concentration suggests the need to apply even higher concentrations,

or more frequent applications, of EPNs in future. Some researchers have proposed the period of pretreatment (in terms of applications made prior to the reproduction of fungus gnats) as being the most effective time during which to apply the EPNs. However, such applications might not be possible, especially for environments that are already infested with fungus gnats. One option for solving the problem of multiple fungus gnat generations on the farm could be to break the reproduction cycle through the chemical control of adult fungus gnats (Vänninen, 2003), followed by repeated nematode applications, which might only be justifiable in the case of a high-income cash crop.

The local EPN, *S. yirgalemense,* which was tested for its potential to control both an artificially introduced, and an established, fungus gnat population, showed high potential for use in fungus gnat management programmes for local farmers. The fact that the species concerned was able to perform well in a local greenhouse, where average temperatures exceeded 25 °C, further supports its ability to be incorporated in fungus gnat management by local growers, in an area where the average temperatures exceed 25 °C. However, even after doubling the recommended concentration of EPNs, the fungus gnat populations quickly restored to their starting numbers after approximately three weeks. Such a phenomenon further emphasises the short life cycle of the fly, as well as the importance of pretreatment or numerous, and consecutive, applications of EPNs, to bring the number of fungus gnats to a level where the flies are not able to cause economic damage.

The soil temperatures in the current study went down to 18 °C at night and did not exceed 31 °C during the day. The medium concerned buffered the nematodes against the prevailing high temperatures, creating optimum conditions in which the IJs could thrive. The ambient temperature is one of the important abiotic factors affecting the efficacy of EPNs. Whereas some EPNs are more adapted to functioning well in higher temperatures, others are more adapted to functioning well in lower temperatures. For example, *S. feltiae*, which has, heretofore, predominated the control of fungus gnats, has been cited to experience biological constraints at relatively high temperatures (Gouge & Hague 1995a, b; Jagdale *et*

al. 2004). Gouge & Hague (1995a) tested different steinernematid and heterorhabditid species to determine their infectivity and insecticidal capacity on sciarid flies. In their study, *S. feltiae*, though being the most effective for controlling sciarid species, became less effective at relatively high temperatures. The UK isolates of *S. feltiae* were also shown to be more effective against UK sciarids than were the other EPN isolates tested from other European countries (Gouge & Hague 1995a). In another study, Jagdale *et al.* (2004) indicate that *S. feltiae* gave better results in a growth chamber (22 °C), achieving a higher control efficiency of 73 % to 80 %, as compared to their achievement of only 34 % to 41 % in a greenhouse, where the temperatures were generally higher (above 25 °C). *Steinernema yirgalemense* was, thus, found to be more suitable for such greenhouses, where the temperatures were relatively high. During their laboratory trials, Katumanyane *et al*. (2017b) found that *S. yirgalemense* was able to cause higher than 80 % mortality of *B*. *impatiens*, both at 25 °C and at 30 °C.

Future studies should consider different factors that might affect the optimum field performance of *S. yirgalemense*. These can include the pretreatment of substrates, the use of higher concentrations of EPNs, multiple, but timely, applications of EPNs, and such other factors as pretreatment with cadaver application, versus aqueous drench application of the EPN species.

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Chapter 5

Conclusion

Bradysia spp., commonly known as dark-winged fungus gnats, mushroom fly, and nuisance flies, are some of the major pests that are capable of causing significant economic losses in crops in protected environments. *Bradysia* spp., which are known to have a global distribution, have recently been found in South Africa in association with major tree nursery beds in the Mpumalanga and Kwazulu-Natal provinces. The species identified was *Bradysia impatiens*, which is regarded as an introduced species. In the course of the current study, the same species was identified in association with many other greenhouse crops in the Western Cape province, including different herbs, cucumbers, tomatoes, mushrooms, blueberries and chrysanthemums. From the surveys conducted, it was concluded that *B. impatiens* is an established covered crop pest in the Western Cape. *Lycoriella sativae* Johannsen, was identified, for the first time, as pest on mushrooms, in South Africa and the Afro tropical region. However, the surveys concerned were targeted at a limited number of greenhouses, thus more surveys are needed to establish the occurrence, and the extent, of fungus gnat infestations, both in and beyond the borders of the Western Cape, focusing on protected crops.

Although various management strategies are available for fungus gnat pests, growers have mostly approached control through the use of chemical insecticides, which, unfortunately, are characterised by careless and abusive use. Even though some chemical insecticides are labelled for use against fungus gnats, currently no pesticides are registered for the control of fly pests on herbs. Biological control using entomopathogenic nematodes (EPNs) has been a preferred method of control. The reason for such preference is that fungus gnats are mostly pests of protected crops that are either close to harvest, as is the case for mushrooms and vegetables, or else the application of pesticides is unsuitable, as is the case for houseplants. The use of EPNs is also made possible by all growth stages of

fungus gnats, except for the adult, being soil-dwelling, with such a habitat providing a long window of opportunity for EPN application. The control of fungus gnats using EPNs has, however, been so far dominated by the use of *Steinernema feltiae*. Although *S. feltiae* has a global distribution, it has, to date, neither been isolated from South Africa, nor from the rest of the African continent, except for Algeria. *Steinernema feltiae*, which is known to be a low-temperature-active nematode, has been cited as having biological constraints at higher temperatures that correspond to most greenhouse temperatures in South Africa. Such temperature thresholds create the need to use endemic EPNs that are better adapted to the local environmental conditions. Thus, the overall objective of the current study was to screen different local EPN isolates for their potential to control *B. impatiens* in South African covered production.

Nine EPN species, including eight local EPN isolates, as well as *S. feltiae* imported from e-nema, Schwentinental, Germany, were screened for their pathogenicity against fungus gnats under optimum conditions in the laboratory. The local EPN isolates used included four *Heterorhabditis* species: *H. bacteriophora*, *H. noenieputensis*, *H. zealandica*, and *H. indica,* and four *Steinernema* species: *S. jeffreyense*, *S. khoisanae*, *S. yirgalemense*, and an unidentified *Steinernema* sp. All nine EPNs were tested on fourth instar larvae of *B. impatiens*, with the instar in question having also been determined to be the stage of fungus gnats that is most susceptible to EPNs.

Larvae of *B. impatiens* were obtained by means of establishing a laboratory culture of the fly on a 3:1:1 mixture of pine sawdust, and corn and soy meal. From the results, it was concluded that the flies take approximately three weeks, at 25 °C, to complete their life cycle. The number of eggs laid per single adult female was between 100 and 250. A new phenomenon of egg laying, which comprises the eggs being laid in chains, was observed for the first time for *B. impatiens*. While the cycle is a complete metamorphosis, the larva stage undergoes four larval stages, which are easily differentiated by their body length and size.

The selected nematode species for controlling the *B. impatiens* were expected to have high insecticidal activity, as well as be adapted to the environmental conditions characterised by persistent high temperatures, which are experienced during the summer months in South Africa, and, in general, in protected crop environments. The EPNs were, thus, subjected to screening at different temperatures, with the percentage mortality of *B. impatiens* being recorded in each case. At first, all nine species were screened at 25 °C, with, thereafter, five EPNs being selected and screened at 13 °C and 30 °C.

The results from the EPN screening for pathogenicity against *B. impatiens* confirmed *S. feltiae* as a low-temperature-active nematode, since it best performed at 13 °C, causing a mortality of 82%, whereas none of the local EPN species was able to penetrate, or to cause mortality, of *B. impatiens* at the same temperature. From the screening, all the relatively longer and thicker nematodes, including *S. jeffreyense*, *S. khoisanae*, and a *Steinernema* sp., were unable to infect the fourth instar larvae of *B. impatiens*. In contrast, the smaller and thinner nematodes, *S. yirgalemense*, *S. feltiae*, *H. bacteriophora*, *H. noenieputensis*, *H. zealandica*, and *H. indica*, were all able to infect the larvae of *B. impatiens*, causing significant mortality. Such infectivity was ascribed to the size of the natural openings on the fungus gnat larvae, namely, the spiracles, the mouth, and the anus, which were assumed to be too small to permit nematode entry. Results from the temperature trials showed the local EPNs *S. yirgalemense, H. noenieputensis*, *H. indica* and *H. zealandica* to achieve > 80% mortality, at 25 °C and 30 °C. Such mortality rates supported their potential for use in covered production, where the average temperature normally exceeds 25 °C. However, further laboratory screening should be done using untested and newly isolated local EPNs, especially in the case of smaller EPNs, to determine both their efficacy and their biological suitability for use in the field.

Field application of EPNs requires mass culturing, the optimisation of traits, and the use of standard formulation techniques, which are still a challenge in South Africa. Ongoing studies have shown both *S. yirgalemense* and *H. zealandica* to be some of the local EPNs

that can easily be mass cultured in the laboratory. Other ongoing studies are focused on the use of mass culture and formulation techniques for *S. yirgalemense,* since it has shown a broad host range. Using such techniques will offer an opportunity for growers in South Africa to incorporate local EPNs in their pest management programmes.

From the temperature trials, three local EPNs, *H. zealandica*, *H. indica* and *S. yirgalemense*, were selected, with their lethal dosages against the fourth instar larvae of *B. impatiens* subsequently being determined. The lethal dosage trials showed *H. zealandica* to have the highest lethal capacity, having an LD_{50} and LD_{90} of 2 and 23 infective juveniles (IJ), respectively, in comparison to that of *S. yirgalemense* (8, 65) and *H. indica* (8, 59).

The study that was aimed at establishing the temporal development of *S. yirgalemense, H. zealandica* and *H. indica* in the fourth instar larvae of *B. impatiens* showed that the three species were able to complete their life cycle within one week, and to produce viable IJs. The ability of an EPN to complete its life cycle in a host insect is important, since it ensures the survival and the persistence of the nematode within the growing medium concerned. This was, thus, an advantage to the three species tested. However, it was observed that the food sources from the host were depleted within a few days, with only one generation being attained in the host, and very few IJs being produced for all the EPNs involved. The need can, therefore, be seen for the follow-up inundative application of EPNs, so as to prevent the number of *B. impatiens* from massing to damaging levels.

Heterorhabditis zealandica required relatively fewer IJs for it to kill the larvae *of B. impatiens*, and, keeping other factors constant, it was shown to be the superior candidate, with regards to its pathogenicity potential. However, *S. yirgalemense* was chosen for the field trials, because of its comparably high virulence, obtained during the screening against *B. impatiens*. *Steinernema yirgalemense* is currently the species of choice, with it having already been successfully mass cultured *in vitro* in liquid culture, and formulated on a small scale, which is currently being upscaled to fermenter size.

The field trial had two objectives, of which one was to determine the effect of different nematode concentrations to control an established population of *B. impatiens* in a commercial cucumber greenhouse, while the other was to determine the effect of three different potting media on nematode movement to control fungus gnats. For the first objective of the field trial, the efficacy of three EPN concentrations of *S. yirgalemense*, consisting of 2.5×10^5, 5×10^5, and 10×10^5 IJs per m^2, was determined over a 4-week period. The concentrations were based on the recommended concentration of EPNs in the product nemaplus® (*S. feltiae*) for the control of sciarids. The recommended concentration is of 5×10^5 IJs per m^2. In the current study, doubling the number of nematodes in the recommended dosage reduced the number of fungus gnats by 77% after 14 days, and by 76% after 21 days, suggesting that even higher concentrations of nematodes can be applied. The results also support the recommendation by nemaplus®, which is to double the concentration in case of high sciarid infestation. Nemaplus® also recommends a follow-up application after 14 days. The results of the current study support the recommendation, since the fungus gnat populations were observed to quickly restore to their original levels, after a period of approximately three weeks. This further emphasises the short life cycle of the fly, as well as the importance of the pretreatment and the consecutive applications of EPNs in bringing the number of fungus gnats to a level where the flies are no longer able to cause economic damage.

In a different trial, the same concentration as recommended by nemaplus® was used to test the effect of potting media on nematode movement, for the control of fungus gnats. *Steinernema yirgalemense* was applied to an introduced and known number of fungus gnat larvae. Results from the trial showed that all three different types of potting media, consisting of pine sawdust, coco coir, and a mixture of both bale coir and vermiculite, all had a positive effect on the movement of *S. yirgalemense.* The type of organic substrate used is an abiotic factor that might affect the efficacy of EPNs in the field, since it influences nematode movement, as well as fungus gnat movement and colonisation. Ultimately, growers are

advised to choose the right medium for crop growth, if considering using EPNs. Mortality in excess of 75% was achieved for fungus gnat larvae that were added to the media, as indicated by the number of adult fungus gnats caught on the yellow sticky cards used. On the basis of such results, and keeping the other factors constant, growers can, thus, continue using the same substrates, even if they intend to incorporate EPNs in their fungus gnat management programmes.

The results of the current study show that, under optimum conditions, the locally isolated EPNs, all with smaller IJs, namely *H. zealandica, S. yirgalemense*, and *H. indica*, tend to have great potential for use as biocontrol agents for *B. impatiens*. However, additional research should be undertaken into the effect of timing, concentrations, and nematode application techniques, among other factors, so as to obtain further critical information regarding the field applications of the nematodes. Further studies should look at different factors that might affect the optimum field performance of *S. yirgalemense*. The factors involved could include the pretreatment of the substrates, using higher concentrations of EPNs, and follow-up, but the timely applications of EPNs and other factors, such as pretreatment with cadaver application, versus the aqueous drench application of the EPN species. However, the application of nematodes should not be seen as a stand-alone, but it should, rather, be implemented as part of an integrated pest management system, with sanitation being the most important consideration.